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# THE TRYPANOSOMES OF MOSQUITOES AND OTHER INSECTS.* †

Frederick G. Novy, Ward J. MacNeal, and Harry N. Torrey.‡

*(From the Hygienic Laboratory, University of Michigan, Ann Arbor, Mich.).*

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## INTRODUCTION.

IN the paper on "Bird Trypanosomes"[1] we expressed the opinion, in view of the ease with which trypanosomes can be cultivated in the test-tube, that it is reasonable to suppose the same result may take place in the mosquito. That is to say, the few trypanosomes which chance to be present in the blood sucked up by the mosquito may rapidly multiply in the stomach of the insect and give rise to rich cultures similar in many respects to those met with in the test-tube. This view, based entirely upon the cultural characteristics of the bird trypanosomes, has an immediate bearing, not only upon the question of the transmission of trypanosomes, but also upon that of the relation of flagellates to certain intracellular parasites, as claimed by the late Dr. Schaudinn, and for that reason it was desirable to obtain, if possible, an experimental confirmation.

The obvious procedure would be to allow mosquitoes which had been raised from eggs in the laboratory, to feed on birds having a pure trypanosome infection. On subsequent examination such mosquitoes should show cultural forms in the gut, if the above view was correct. Unfortunately, on account of the difficulty of procuring and keeping alive wild birds the choice of birds for these experiments was limited to the sparrow and canary. In our previous work we had found trypanosomes in sparrows, but only in a very small percentage of the birds examined. We hoped, however, by means of cultures of the bird trypanosome to secure pure infections of sparrows and canaries, and with this object in view, a large number of sparrows were examined during the summer of 1905, microscopically and culturally, but with negative results. The failure to obtain infected sparrows and the inability to work with *Tr. paddae* which, as shown by Thiroux, is admirably adapted for laboratory infection, compelled us to abandon this plan and resort to a different procedure.

The rather common occurrence in birds of trypanosomes, and of cytozoa, suggested a study of the flagellates in "wild" mosquitoes. If our view was correct, then it might be possible to find in the gut of such mosquitoes cultural forms of bird trypanosomes. As will be shown, flagellate infection of mosquitoes is quite common, but the forms met with do not correspond to any one of the numerous strains which we cultivated from birds. Previous observers had already described such organisms in the gut of insects, but their source or nature was far from being understood. They might be derived (1) from the trypanosomes of the blood of birds or other animals; or (2) they might be stages in the life-cycle of cytozoa, as held by Schaudinn; or (3) lastly they might be harmless, non-pathogenic parasites peculiar to the insect and in nowise related to either of the two types mentioned above.

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[1] *Jour. Infect. Dis.*, 1905, 2, p. 303.

It is plain that each of these three possibilities must be considered in order to arrive at a definite conclusion regarding the flagellates found in the gut of sanguivora, such as mosquitoes, flies, fleas, lice, leeches, etc. A mere morphological study is incapable of deciding for certainty between the three conditions. Even when supplemented by animal inoculations, effected either by the bite of the mosquito, etc., or by injection of suspensions of the insects, the result is still open to question. For it is conceivable that the mosquito or other host may have a double or even triple infection, and hence the result obtained (i. e., appearance of cytozoa) may be due to an unrecognized stage and not to the flagellates actually seen. The recent work of Edm. and Et. Sergent on the transmission of the halteridian infection of pigeons affords a demonstration of the existence of such a stage. It is true that in proteosoma infection, and in human malaria this method of investigation has given most satisfactory results, but when applied to flagellate studies it may prove, and undoubtedly has proven, misleading. The relation of cause and effect in such problems can be brought out with the least uncertainty by the aid of pure cultures of the trypanosomes in question, and this method of study, so well established in bacteriology, should be applied as far as possible to the solution of questions in protozoology. By this means we have already shown, contrary to Schaudinn, that the trypanosomes of birds are in nowise related to the hemocytozoa. The applicability of this method of research to insect trypanosomes will be seen from the results of this study.

Our investigations upon the mosquito trypanosomes were made during the summers of 1905 and 1906. They show that the flagellate infection of mosquitoes is of common occurrence, and that several distinct species are to be found. It has been possible to grow these organisms in mixed and even in pure culture, and to show that they are closely related to, and indeed actually are, true trypanosomes. The forms which have been obtained in the culture-tube are similar to, and even identical with, those found in the digestive tube, thus showing that the forms met with *in vivo* are in reality cultural forms, as we have contended. This fact goes to show the absurdity of the contention that cultural forms represent degeneration or involution types. The cultivation method has enabled the differentiation into two species of forms heretofore regarded as belonging to one organism. Furthermore, the inoculation of various birds and mammals with such cultures was not followed by the development of intracellular parasites, or by the appearance of trypanosomes in the blood, which would seem to indicate that the mosquito flagellates are parasites peculiar to this insect.

## PREVIOUS OBSERVATIONS.

Before proceeding to a detailed account of our work, it is desirable to present, as briefly as possible, a résumé of the observations which have been made up to the present time regarding the presence of flagellates in the guts of various insects and also of leeches. It will be seen that flagellate infection of the digestive tube is not limited to the sanguivora, but that it may occur in insects that do not feed or have not fed on animals.

The earliest observations upon the presence of flagellates in mosquitoes were made by Ross, in 1898, in India. Recently (1906) he has called attention to these early studies, and there can be no doubt but that the forms seen by him were the same as those which Léger subsequently described as *Crithidia fasciculata*. In various species of *Culex*, principally *C. fatigans*, he found "sporangia" or radiating clusters (rosettes) of small active bodies, which under water separated into thousands of flagellulae. These were about 8 $\mu$ by 2 $\mu$ in size, with numerous minute black points in their substance, and had a single long flagellum. Another form, which he designated as amoebulae, he now considers as transitional, dividing forms corresponding to the truncated *Crithidia*. Apparently, the long *Herpetomonas* which will be described later was not encountered. The forms mentioned were also observed by him in *Anopheles*, but not in *Stegomyia*. They occurred in the larva, pupa, and imago; at times, in a considerable proportion of the insects examined. It is interesting to note that Ross during his work on Proteosoma found the flagellates but once in mosquitoes, although these were fed on birds many of which contained Halteridium as well as Proteosoma. This fact, as well as their presence in all three stages of the insect, goes to show, as Ross points out, that they had been already present in the insects before these were fed on the blood. He very correctly concludes that there is little reason for supposing that the *Crithidia* are developed from cytozoa, and he expresses the belief that they are derived from organisms contained in the intestines of the larva and probably swallowed by it. He further points out the probability that Schaudinn's mosquito flagellates were merely *Crithidia* which were already present in the insects with which he worked.

The next observation is that of Durham, who, in 1900, examined a specimen of *Stegomyia fasciata* which had fed the night before on a small bat. The blood in the mosquito contained abundant trypanosomes whose shape was quite different from the usual ones in rats, nagana, etc. The presumption was that these trypanosomes came from the bat, but this, owing to its death and rapid decomposition, could not be verified with certainty. Although flagellates, coccidia-like bodies, etc., were found from time to time in the eighty mosquitoes which were dissected, this was the only instance in which trypanosomes were found. Nothing further is stated regarding the flagellates mentioned.

Chatterjee, in 1901, while dissecting different *Anopheles*, found in the abdominal cavity of one of them an organism which appeared to him to be closely related to the trypanosome of surra. It was actively motile and possessed a fine flagellum, longer than the body, and an oval nucleus. Not having access to the original paper, it is impossible to say whether this form belonged to *Crithidia* or *Herpetomonas*.

In the same year Christophers noted the presence of swarms of a flagellate organism in the rectum and throughout the hind- and mid-gut, in a large proportion of *Anopheles* and *Culex*. The illustration given by him (Ref. 13, Fig. 3, Plate V) is suggestive of *Crithidia*. The presence of these parasites is also mentioned by Stephens and Christophers.[62] In this connection it may be stated that from recent private communications of Captain Patton and also of Captain Christophers we learn that a large percentage of mosquitoes at Madras have *Herpetomonas* and *Crithidia*. Christophers writes that he has found developmental or immature forms of *Herpetomonas*, clustered about the pylorus, in the larvae of *C. fatigans*.

An important study on the flagellates of mosquitoes was published by Léger in 1902. In the gut of *Anopheles maculipennis* he found a peculiar flagellate, presumably the same as that observed previously by Ross and Christophers, which on account of certain characteristics he placed in a new genus, to which he gave the name *Crithidia*. He classed it among the Cercomonadina, intermediate between *Herpetomonas* and *Trypanosoma*. The distinction between the two genera, *Herpetomonas* and *Crithidia*, was based largely upon the difference in size of the adult, free, or monadian forms. It will be shown presently that Léger failed to recognize the possibility of double infection in insects, and that he has at times included in one description two distinct species.

In his critical study of the blood flagellates, Lühe points out that the difference in size of the adult, free, or monadian forms is hardly sufficient to justify the creation of a new genus. Lühe, however, would characterize the genus *Crithidia* as possessing a single flagellum with the blepharoplast situated near the nucleus, whereas the true *Herpetomonas* has a double flagellum (as described by Prowazek for *H. muscae-domesticae* and *H. sarcophagae*) and a terminal blepharoplast. This characterization of the two genera is open to question, as will be shown later on. It should be borne in mind, however, that the distinctions between *Trypanosoma*, *Herpetomonas*, and *Crithidia* are at the best highly fragile.

It is interesting to note that Léger raised the question, in view of the way in which *Anopheles* fed, and the analogy of the elongated forms to trypanosomes, whether the

*Crithidia* did not represent a developing stage of some flagellate hematozoon, more especially the trypanosomata of vertebrates. This question is as yet unanswered, although it is probable that the *Crithidia* are parasites peculiar to the mosquito and are not immediately derived from the trypanosomes in the blood of other animals. As will be seen, Schaudinn has endeavored to show that these, or very similar forms, represented stages in the development of the *Halteridium*.

Léger designated the flagellate found by him in Anopheles as *Crithidia fasciculata*. He described two types with transitional forms one to the other. The first, or gregarine-like, or resting form, is more frequent. It appears as a short, thick oval, resembling a grain of barley. It is slightly flattened, and the anterior end, which carries a whip as long as the body, is truncated. These smaller forms measure 3–4 μ in length, but may attain 6–8 μ. The anterior end is slightly curved or may be indented. The body is hyaline or faintly granular, and at times shows one or more clear spots resembling vacuoles. The whip starts at the centrosome or blepharoplast which lies in front or to the side of the nucleus. The division is longitudinal and corresponds to that of trypanosomes. Rosette formation is common, and the small cells may be united in masses or in radiating bundles which are attached to the intestinal wall.

The second, termed the monadian or free form, is an elongated cell, 8–14 μ in length, and resembles exactly small trypanosomes. The anterior pointed end is drawn out along the whip, and the cell shows a rudimentary undulating membrane. As mentioned above, Léger held that the length of the free form afforded the chief distinction between this genus and that of *Herpetomonas*.

Léger and Duboscq in the same year found this organism, either as the free cells or in massive rosettes, in the intestines of *Anopheles* larvae, as well as in hibernating adults.

The work of Schaudinn, in 1904, is of especial importance, for, no matter what may be the final interpretation of his observations, it has served to call attention to the flagellates present in the digestive tract of insects and other sanguivora, and in so doing has stimulated inquiry, not only as to the relation of these forms to the blood protozoa, but also as to the mode of transmission of the pathogenic protozoa in general. As is well-known, he described the formation of trypanosomes and so-called spirochetes in the common mosquito, *Culex pipiens*, which had previously fed on owls infected with intracellular organisms (Halteridium and Leucocytozoon) and trypanosomes. In view of the the fact that a summary of this extensive paper was given in our paper on "Bird Trypanosomes," it is hardly necessary to repeat it here. It was pointed out in that paper that the mosquito flagellates of Schaudinn were probably derived from the bird trypanosomes which had multiplied in the digestive tube of the insect. As will be seen, the present study offers another and more plausible explanation of the origin of Schaudinn's flagellates.

It is important to note, as Schaudinn himself has pointed out, that the forms of *Trypanosoma noctuae*, as found and described in the mosquito, show the greatest resemblance to the *Crithidia* of Léger. Indeed, because of the gregarine-like, resting condition and the mode of multiplication, he inclined to the belief that the *Crithidia* represented a developmental stage of a closely related parasite. Notwithstanding the fact that Schaudinn recognized this similarity, there is no evidence, as Ross rightly asserts, that he excluded the presence of *Crithidia* in the mosquitoes used in his experiments. It is a striking fact that, while he refers to Léger's observation on *Crithidia*, he makes no mention of the presence of the same or similar forms in the *Culex pipiens* with which he experimented, and which were presumably captured at large. From what is now known regarding the distribution of *Crithidia* and *Herpetomonas* in mosquitoes, we must recognize a grave source of error which Schaudinn apparently failed to take into consideration. It will be remembered that he was able to find trypanosomes in only about 10 per cent of the mosquitoes used in his experiments. As will be seen, we have found flagellates in as many as 15 per cent of the mosquitoes captured during one season. This fact itself is very suggestive of the nature of the organisms observed by him. It is our belief that Schaudinn's *Tr. noctuae* is in nowise related to the owl halteridium, and that it either corresponds to *Crithidia fasciculata* or to a mixed *Crithidia* and *Herpetomonas*. Furthermore, the *Spirochaeta Ziemanni* which he later recognized as a trypanosome and not a true spirochete, would seem to correspond to a *Herpetomonas* like our *Tr. culicis* or to *Tr. avium* were it not for the recent observation of Töpfer regarding the presence of spirochetes in owls. This work, as will be shown later, throws new light upon the nature of *Sp. Ziemanni*.

In the same year (1904) Edm. and Et. Sergent, working in Algeria, repeated the work of Schaudinn and obtained essentially the same results.[1] That is to say, flagellates were found in 28 per cent (14 out of 50) of *Culex pipiens* which were examined 36–48 hours after having been fed on an owl (*Athene noctua*), infected with halteridia. This large percentage of infected mosquitoes, as compared with Schaudinn's 10 per cent, is of no special significance, since we have repeatedly met with small batches of "wild" mosquitoes in which one third or more showed flagellates. The Sergents state explicitly that they had never met with similar forms in the stomachs of mosquitoes which had not fed on infected blood.

This general statement, in view of the widespread natural infection of mosquitoes, is difficult to understand unless it be assumed that these control insects were examined directly—that is, without having been fed upon a clean animal. It is perfectly obvious that a rigid control test demands that the mosquitoes be fed upon the blood of a clean, or non-infected bird, or even mammal, in order to allow the flagellates, if any are present, to multiply. The fact that the mosquitoes used in these experiments were raised from larvae in the laboratory does not, as is now known, exclude a natural flagellate infection of the adult insect. This fact they themselves recognized in the following year, as will be shown presently.

With the mosquitoes which had fed upon the infected owls the Sergents were able to produce a halteridium infection in young owls which had been raised in the laboratory and were presumably free from parasites. Thus, an owl injected with the stomach contents (containing trypanosomes) of a mosquito that fed on an infected bird two days before, developed halteridia 11 days later. Similarly, a second owl which received the stomach contents of two mosquitoes showed halteridia three days later. On the other hand, a third owl which received an injection of the stomach contents of three mosquitoes failed to become infected (!). In another experiment six mosquitoes, fed a month before on an infected bird, were allowed to bite a young owl, which soon developed an infection. Two other owls bitten by these same mosquitoes or by others, failed to show halteridia.

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[1] The forms which the Sergents identified as "indifferent" trypanosomes measured 25 μ in length by 3 μ in width. The larger ones were 36 μ long. This is given as the total length including the flagellum, the latter being as long as the body. The "male" trypanosomes were small, on an average 14–15 μ in length, and this presumably includes the whip. The deeply staining, granular "female" forms were rare. They note a slight difference between their trypanosomes and those of Schaudinn in that their forms were "plus effilés, moins renflés" than those figured by the latter. This fact, taken into consideration with their description of the indifferent, male and female, forms, makes it probable that they were dealing with *Herpetomonas*, probably the same as our *Tr. culicis*. On the other hand, Schaudinn's figures (Figs. 1, 2, and 4) show a rounded posterior end, and in this respect resemble *Crithidia;* in other regards, like the presence of an undulating membrance and posterior bodies, they suggest a *Herpetomonas*. The *Herpetomonas* only rarely shows an enlarged, rounded posterior end, as will be shown later in connection with the involution forms of *Tr. culicis*.

The Sergents looked upon their work as a crucial experiment supporting Schaudinn's view as to the relation of *Tr. noctuae* to the halteridium. It must be confessed, however, that these tests were by no means as rigid as they supposed; for, although they employed mosquitoes raised from the larval stage and made use of young birds taken from the nest, and found presumably to be free from infection, they clearly overlooked several possible sources of error. In the first place, the owls upon which the mosquitoes fed may have been infected with trypanosomes as well as halteridia. They themselves recognized the presence of a trypanosome in 1 out of the 3 or 4 infected owls with which they worked, and it is not unlikely that this flagellate was present in the others, though in very small numbers. It is therefore possible that the mosquitoes which fed upon these owls developed a double infection, trypanosomal and halteridian, the latter being readily transmitted by an unrecognized stage, whereas the former gave rise to a "culture" of the flagellates in the digestive tube. An error of this kind can be obviated by demonstrating that the birds are *culturally as well as microscopically* free from trypanosomes.

A second and perhaps even more important factor which must be taken into consideration is the fact of the not infrequent presence of trypanosomes in the larval and pupal stages, and hence in adult mosquitoes before these have fed upon the infected bird. It is evident that the use of mosquitoes which have been raised in the laboratory from the eggs does not insure the exclusion of flagellate infection of the intestinal tract of these insects.[1] This fallacy can be guarded against by means of a control experiment in which a large enough number of "raised" mosquitoes are fed upon non-infected animals. In this way it should be possible to show whether or not the percentage of flagellate infection is greater in the mosquitoes which fed upon infected birds, and whether such flagellates are identical with or different from those naturally present in the stomach.

A third and equally important objection is based upon the apparent absence of control inoculations with the stomach contents, free from trypanosomes, of mosquitoes fed on infected birds. In Schaudinn's work 90 per cent of the mosquitoes thus fed showed no flagellates, while with the Sergents the percentage was not as high, only 72 per cent. What would be the result if the stomach contents of these mosquitoes was injected into clean owls? There is no record that such experiments were made; and yet the need of a control test of this kind is perfectly obvious; for, if the halteridian infection is due to something other than flagellates, it should be possible to secure positive results with such trypanosome-free mosquitoes.[2] On the other hand, the failure

[1] As will be seen from their work of the following year, reference to which is given below, they succeeded in finding a *Herpetomonas* in 11 per cent of mosquitoes raised from the larval stage in the laboratory.

[2] The possibility of the presence of an unrecognized stage of halteridium in the mosquitoes is seen from the recent work of the Sergents on the transmission of this parasite to pigeons. After feeding some Hippoboscides (*Lynchia maura*) on infected pigeons in Algeria, the insects were taken to Paris and placed on clean pigeons. The latter became infected after a period of incubation of 34–38 days. Intravenous injections of suspensions of *Lynchia* caused infection after 28–29 days; and a Berkefeld filtrate infected after 36 days. This long period of incubation, when compared with that noted in owls after injection of mosquito trypanosomes, lends color to the belief that these birds had an unrecognized latent infection. Trypanosomes were apparently not present in the infected *Lynchia*.

to obtain infection in such an experiment would be an argument in favor of Schaudinn's views.

Another source of error which must be rigorously guarded against is the possibility of the existence of a latent halteridian infection in the owls seemingly free from parasites. The infection of owls and other birds very commonly takes place before they leave the nest. This fact was first pointed out by Danilewsky, and we have repeatedly made similar observations. Hence, nestlings cannot be said to be free from infection unless repeated examinations are made *extending over a period of several weeks* to allow for the maximum period of incubation. A very good illustration of latent infection is sometimes observed in sparrows which receive an injection of proteosoma. Although at first apparently free from parasites, they may develop halteridia in the course of a week, along with the proteosoma. The halteridia were not introduced by the injection, for it is not possible to infect birds in that way. The statement of Schaudinn that the halteridian infection of owls can be transmitted from owl to owl by injections of the infected blood, "was ja schon lange bekannt ist," is open to question, as previous workers, employing, it is true, other kinds of halteridia, have not been able to induce an infection by means of blood injections. In view of this fact, it is probable that the apparently successful inoculations with infected blood (and with mosquitoes) may be due at times to the lighting-up of a latent infection. (See also p. 240 and footnote p. 259.)

Although unconfirmed, except as mentioned above, still Schaudinn's views regarding the nature of the mosquito flagellates have been accepted by most zoologists. Some, indeed, have gone as far as to interpret the work done on the owl as applying to all trypanosomes, and in this they have gone beyond the position taken by Schaudinn himself; for he realized, as we know from his letters, that there were trypanosomes without an intracellular stage, and that there were halteridia which had no trypanosome stage. As a matter of fact, the whole question has narrowed itself down to the parasites of *Athene noctua* and their relation to the mosquito flagellates. It is not a question of a broad, general biological principle.

The work of the Sergents is of further interest, inasmuch as they found in the Malpighian tubes of mosquitoes (three out of 17) which fed on owls infected with *H. Ziemanni* the spirochetal forms described by Schaudinn. These flagellates, which they describe as being 25–30 $\mu$ long and 1 $\mu$ wide, are clearly different from the true spirochetes, which scarcely exceed 0.2 $\mu$ in width. Similar forms were not found in 52 Culices from the same brood as those used in the experiment; but whether these control mosquitoes were fed on non-infected birds is not stated. Repeated attempts to inoculate a barn owl, *Strix flammea*, either by injection of these flagellates or by the bites of mosquitoes, failed.

The measurements as given by the Sergents for *Sp. Ziemanni* agree perfectly with those of the long Herpetomonad form (*Tr. culicis*) which will be described later. This statement does not necessarily imply that the forms described by Schaudinn correspond to this trypanosome, for, as a matter of fact, his illustrations of *Sp. Ziemanni* (see his Fig. 17) show forms which have the blepharoplast posterior to the nucleus, and hence have a very long undulating membrance. Such is not the case with *Tr. culicis*. The forms mentioned, however, do resemble, remarkably, the free-swimming, long type of *Tr. avium*, as we have shown in the paper on "Bird Trypanosomes" (Plate 10). As to the possibility of the presence of true spiochetes see footnote p. 259.

Still more recently (1906) the Sergents have reported upon the presence of a *Her-*

*petomonas* in 13 out of 119 (11 per cent) *Culex pipiens* and in one *Stegomyia fasciata* which were raised from larvae in the laboratory. These mosquitoes, it should be noted, though fed either on infected owls or on clean canaries, showed as large a percentage of infections as was obtained by Schaudinn. They designate this organism as *Herpetomonas algeriense*, and describe two forms, motile and non-motile. The motile form was 12 μ long and 2.5 μ wide, and was provided with a whip which was 3.5–6 μ long. The large transverse centrosome was always posterior to the nucleus and about 0.56 μ from the broad rounded end. The non-motile forms were round, about 5.5 μ in diameter, and had a long, free flagellum measuring even 17 μ in length. Although the mosquitoes fed in part on owls infected with halteridia and *H. Ziemanni*, they do not mention finding the same trypanosomes as in the previous year. (See footnote p. 229.)

They also report on the presence of a very narrow flagellate in the digestive tube of the larvae of several *Anopheles* and of one *Culex;* and also of one nymph of *A. maculipennis*. The body averaged 16 μ in length and 3.5μ in width, and was provided with a long free flagellum which measured 24 μ. They regard this organism as a *Herpetomonas* resembling the *H. jaculum* which Léger found in *Nepa cinerea*, and they suggest that possibly the latter obtains its infection by feeding upon the mosquito larvae. It will be seen from these observations of the Sergents that they have described four kinds of flagellates in mosquitoes, two of which they identify with the forms of Schaudinn.

Summing up the investigations heretofore made regarding the flagellates of mosquitoes, it will be seen that two distinct types have been recognized, *Crithidia* and *Herpetomonas*, and that each of these is represented by one or more species. Exactly similiar forms have been found in the digestive tract of other insects, some of which, it is worth noting, are not sanguivora.

The following list of the species described up to the present time may not be without interest:

| | |
|---|---|
| *Crithidia fasciculata*, Léger, 1902 | In the gut of adult *Anopheles* and *Culex;* also in larvae and pupae. |
| " *campanulata*, Léger, 1903 | In intestine near Malpighian tubes in larva of *Chironomus plumosus*. |
| " *minuta*, Léger, 1903 | In mid- and hind-gut of *Tabanus tergestinus*. |
| *Herpetomonas muscae-domesticae*, Burnett, 1851. | In the common house-fly; also in *Homalomyia scalaris*, *Pollenia rudis*, *Theicomyza fusca* (Léger). |
| " *Bütschlii*, Kent, 1881 | In intestine of *Trilobus gracilis*. |
| " *jaculum*. Léger, 1902 | In mid-gut of *Nepa cinerea*, adult and larvae. |
| " *gracilis*, Léger, 1903 | In Malpighian tubes of larvae of *Tanypus*. |
| " *Lesnei*, Léger, 1903 | In mid-gut near Malpighian tubes of *Dasyphora pratorum*. |
| " *subulata*, Léger, 1904 | In gut of *Tabanus glaucopis* and *Hematopota italica*. |
| " *sarcophagae*, Prowazek, 1904 | In gut of meat-fly, *Sarcophaga hemorrhoidalis*. |
| " *bombycis*, Levaditi, 1905 | In butterfly of silkworm, *Bombyx mori*. |
| " *algeriense*, Edm. & Et. Sergent, 1906 | In gut of *Culex pipiens* and *Stegomyia fasciata*. |
| *Trypanosoma noctuae*, Schaudinn, 1904 | In gut of *Culex pipiens* as supposed stage of *Halteridium noctuae*. |
| " (*Spirochaeta*) *Ziemanni*, Schaudinn, 1904 | In gut of *Culex pipiens* as supposed stage of *H. Ziemanni*. |
| " *Grayi*, Novy, 1906 | In gut of *Glossina palpalis*. |
| " *Tullochii*, Minchin, 1906 | " " " " " |
| " *culicis*, N. Sp | In gut of various culices. |
| " *Christophersi*, N. Sp | In gut of dog-tick, *Rhipicephalus sanguineus*. |

The unnamed herpetomonas forms observed in *Culex*, *Anopheles*, and *Stegomyia*, and also that met with in *Stomoxys* by Gray, obviously cannot be included in the above list. This is also the case with a very small flagellate, one-fourth to one-fifth the diameter of a red blood cell, which Ziemann found in the ovaries of *Chrysops dimidiatus*, a biting fly in Camerun. It may be stated further that Rogers has recently designated the human parasite, *Piroplasma donovani*, as Herpetomonas of Kala-azar. The flagellate which Dutton and Todd found in two out of fourteen house mice in Senegambia, and which they compared with *H. Bütschlii*, is probably identical with *Tr. duttoni* which Thiroux later described in the mice of Senegal. It may further be stated that in *Nepa* Léger found a short oval flagellate about 6 $\mu$ long, which he designated as *Otomonas tremula*.

The flagellates recently (1905) described in the gut of the sheep louse (*Melaphagus ovinus*) by E. Pfeiffer have been compared with *Crithidia* and *Herpetomonas*. They may be looked upon as "cultural" trypanosomes, as they show the blepharoplast anterior and close to the nucleus, while the undulating membrane is indicated with reasonable certainty in some of the illustrations given, especially in his Fig. 7. The intestinal epithelial cells were covered with rows of the resting forms, and, in addition, typical rosettes were observed. Unfortunately, no examination of the blood of the sheep from which the lice were taken is mentioned, and presumably was not made.

It is a well-recognized fact that *Tr. Lewisi* is carried from rat to rat by fleas and lice. The transmission by fleas was first established by Rabinowitsch and Kempner, although they were unable to detect the flagellates in the fleas by direct examination. In lice which have recently fed on rats infected with *Tr. Lewisi* this organism can be readily seen, and by placing freshly infected lice on a rat MacNeal was able to secure an infection. The fact that similar experiments failed, with lice containing more or less completely digested blood, goes to show that these insects do not play an active part as hosts, but are mere vectors or passive carriers of the parasites in much the same way as in the case of the tsetse fly.

According to the recent work of Prowazek (1905), the rat louse (*Haematopinus spinulosus*) is an intermediary host, inasmuch as he found trypanosomes, though in variable numbers, in almost all of the lice which fed on infected rats. The parasites were usually present in the stomach; twice they were seen in the Malpighian tubules, and even in the circulating blood, and once in an egg. That the flagellates were actively multiplying in the mid-gut was evident by the presence of divisional forms and of large rosettes or groups which covered the lower end of the stomach wall, especially in the region of the Malpighian tubules. The illustrations which he gives (such as Fig. 55) show undoubted "cultural" forms, since the blepharoplast is anterior to the nucleus and the flagella in the rosettes are directed centrally. Crithidia-like bodies with short flagella, as well as smaller involution forms, without whips, were found. The latter forms were either free in the gut or wedged deep between the cells. He described male and female forms, although, as in the case of *Herpetomonas muscae*, the difference between the two was not very marked.

Prowazek identifies these flagellates of the louse with *Tr. Lewisi*, but this is by no means proven, since he was unable to infect rats by placing upon them presumably infected lice. If the multiplying flagellates of the louse actually represent stages of the cultural form of *Tr. Lewisi*, it should manifestly be possible to produce an infection either by injecting the stomach contents of such lice after they had fully digested the blood which they had taken up, or by allowing them to feed upon clean normal rats. Our own experiments, as mentioned above, indicate that the transmission is effected by *lice which have just fed on an infected rat* and not by those which are void of fresh blood. While, therefore, it is quite reasonable to believe that the ingested trypanosomes multiply in the gut, it is clearly not proven. Although we have carefully examined Prowazek's memoir, we have not been able to find any statement to show that lice taken from non-infected rats were free from these flagellates. Control examinations of this kind are as essential here as in the work upon mosquitoes. In the absence of such controls and of positive infection experiments, the possibility remains open that these organisms are mere intestinal flagellates of the louse in nowise derived from *Tr. Lewisi.*

In support of this view, and to illustrate the need of controls, we may cite some experiments in which mosquitoes were allowed to feed on rats infected with *Tr. Lewisi* and *Tr. Brucei.* In the stomach contents of these insects the original blood trypanosomes could be detected for 24 to 36 hours after the infective feed, as will be shown later. At times, *Crithidia* and *Herpetomonas* were present in these insects, and, were it not known that these belonged to the mosquito, they might well have been taken to represent multiplication forms of the blood trypanosomes.

As bearing upon the work of Prowazek should also be mentioned the observations of Nabarro and Greig, Gray and Tulloch, and of Koch regarding the presence of flagellates in the stomach of different species of tsetse flies. These organisms were regarded as multiplication forms of *Tr. gambiense* and *Tr. Brucei,* but the later studies of Minchin, Gray and Tulloch, and of one of us (Novy) show quite conclusively that such is not the case, and that these flagellates (*Tr. Grayi* and *Tr. Tullochii*) are mere harmless parasites of the intestinal tract analogous to those met with in mosquitoes. During the past year Koch himself has recognized the absence of any relation between these flagellates and *Tr. gambiense.* He now inclines to the belief that the tsetse flagellates are derived from the trypanosomes in crocodiles, on account of the similarity which they bear to the cultural forms of the latter.

Another instance of the presence of flagellates in insects is a recent observation made by Balfour (1906). While searching for a developmental cycle of *Hemogregarina Balfouri* in the flea (*Pulex cleopatrae*) which infects the Sudan jerboa, he found a flagellate which occurred in rosettes, or in slightly active forms, and as non-motile gregarines or amoebulae. He regards these organisms as being probably closely related to Léger's *Crithidia fasciculata,* and he further considers it likely that they are "merely parasites of the flea itself" and are unrelated to the hemogregarines of the jerboa. The fact of their presence in fleas fed on non-infected gerbils is important evidence on this point, and is in accord with the observations already noted.

It may further be said that Pricolo has found trypanosomes in fleas taken from mice (*Mus musculus*) which were infected presumably with *Tr. Duttoni.* The significance of the trypanosomes found in cattle and dog ticks will be discussed later.

While on a priori grounds one might be led to believe that the trypanosomes of vertebrates multiply in the digestive tract of insects, the fact remains that up to the present time no positive evidence has been brought forward in support of that view. There is, of course, no doubt as to the fact that trypanosomal infections are spread through the agency of insects, but these seemingly act the part of mere vectors and not of active hosts. It is probable that an exception to this statement is found in the case of kala-azar of India. The parasite of this disease, known as *Piroplasma donovani,* according to private communication from Captain Patton, develops in the gut of the common bedbug into flagellate forms, which then multiply and resemble the *Crithidia* of mosquitoes. This, it may be added, is apparently the only instance apart from the work of Schaudinn on the parasites of the owl, where an intracellular (leucocytic) parasite actually develops into a flagellate stage.

On the other hand, there is one group of organisms, the leeches, where the evidence as to the multiplication of trypanosomes in the gut is quite incontestable. The presence of flagellates in leeches (*Piscicola* and *Pontobdella*) was first noted by Leydig (1849) and later by Labbé in *Haemopis sanguisuga* (15 days after removal from frogs), and it was on account of these observations that Doflein (1901) concluded that the fish trypanosomes were probably transmitted by means of these sanguivora.

Keysselitz was the first to approach the question methodically. Early in 1904 he showed that the leeches were to be considered as intermediate hosts, but the full results of his investigations were not published until 1906. He found a large percentage of leeches (*Piscicola geometra*), captured at large, to harbor in their stomachs variable quantities of flagellates, which because of their morphological characteristics he identified with trypanosomes and trypanoplasmes. Owing to the difficulty of obtaining leeches free from flagellates, he recognized the possible error in employing these in feeding experiments upon fish. He correctly reasoned that, though the fish flagellates would be thus introduced into the leech, it would be impossible to determine whether the multiplication forms observed belonged to them or were derived from the organisms already present in the leech. Hence, in order to obtain unquestionable results, it would be necessary to use leeches that had been raised from the egg and found, by control feeding experiments, to be actually free from flagellates. In his paper he describes, at length, the multiplication forms of trypanoplasmes, but the origin of these is not quite clear. He failed to produce an unquestionable infection by other injections of the stomach contents of the leech or by allowing the latter to feed upon the fish. It is probable that this failure was due to the use of non-susceptible species or such as had an acquired immunity. As will be shown below, Brumpt has succeeded in infecting fish, frogs, and eels by means of leeches.

Billet in 1904 also presented observations going to show that the *Tr. inopinatum* of the Algerian green frog multiplied very easily in the digestive tube of a leech (*Helobdella algira*). Like Keysselitz, however, he was unable to infect frogs either by injection of blood or by the use of infected leeches. Both results, it may be added, have been accomplished since then by Brumpt.

About the same time Léger experimented with loaches which harbor two kinds of flagellates, trypanosomes and trypanoplasmes. The intestinal contents of leeches (*Piscicola*), about four days after these had fed on loaches infected with *Tr. barbatulae*, showed numerous flagellates presenting the three types of Schaudinn. Similarly, leeches (*Hemiclepsis marginata*) placed on fish infected with *Trypanoplasma varium* showed in a few days numerous, small, almost filiform trypanoplasmes in addition to trypanosome-like forms. These observations confirmed the previous results of Brumpt, who noted the presence of prodigious quantities of trypanosomes, showing gregarine and herpetomonad forms, in *Hemiclepsis* taken from fish.

The several studies of Brumpt are of especial importance, as they clearly point to the rôle of leeches in the transmission of flagellates. He brought out the interesting fact that certain species of trypanosomes and trypanoplasmes develop in a given species of leech (*Hemiclepsis marginata*), but not in another (*Piscicola*), while others develop in the latter and fail to do so in the former. He made use of this fact in differentiating a large number of species of trypanosomes and trypanoplasmes. The flagellates found in the stomach and intestines present elongated, herpetomonad, or "cultural" forms, which are sufficiently characteristic to permit the recognition of species. In these forms, as with most of our cultural forms, the blepharoplast is anterior to the nucleus.

According to Brumpt, the *Tr. granulosum* of the eel when sucked up by the *Hemoclepsis* gives rise in a few hours, in the stomach, to pyriform or crithidia-like bodies, which then pass into the intestine, where they persist in the herpetomonad form for months. Hence he regards the latter as the ancestral form preceding the sanguicolous stage. Such leeches when placed on "clean" eels gave a positive infection in from four to six days. Contrary to Keysselitz, he was able to infect two carps, two chubs (chabots), and one cottus by placing upon these leeches taken from the same species of infected fish.

Similarly, Brumpt has shown that the *Tr. inopinatum* of the Algerian green frog when taken up by a small leech (*Helobdella algira*) rapidly assumes the herpetomonad form, and that such leeches when placed on a frog, even as late as a month after the first feeding, produce a severe and even fatal trypanosomal infection after a period of incubation of from eight to 10 days. Unlike Billet, who, influenced by Schaudinn's work, was inclined to consider the leech trypanosome as a stage of the intracellular *Drepanidium*, he points out that there is no relation between these flagellates and the hemogregarines of frogs.

Lastly, it is in order to emphasize, as Léger has done, the fragile distinctions which exist between *Crithidia*, *Herpetomonas*, and *Trypanosoma*. The first two genera are supposed to have no undulating membrane, and yet a rudimentary one is ascribed by Léger to *H. subulata* and to *C. fasciculata*. On account of the proximity of the centrosome to the anterior end, it is evident that the undulating membrane must be but feebly developed, and hence cannot be readily seen. This is equally true of many of the cultural trypanosomes, and for that reason the failure to recognize the presence of an undulating membrane does not, or should not, exclude an organism from the trypanosome group.

Another difference which has been insisted on is the position of the centrosome or blepharoplast which, in *Crithidia* and *Herpetomonas*, usually lies by the side of, or anterior to, the nucleus, while in the blood trypanosomes it occupies a posterior position. This distinction, however, disappears when the cultural forms of trypanosomes are compared, as they properly should be, with these insect flagellates. Such comparisons are in order, for, as will be shown, the *Crithidia* and *Herpetomonas* present identically the same form in the test-tube as in the digestive canal of the insect. The native flagellates found in the gut of insects, therefore, are cultural forms, and as such are directly comparable with those of the blood trypanosomes. Now, in cultures of *Tr. Lewisi*, *Tr. Duttoni*, *Tr. rotatorium*, *Tr. Mesnili*, *Tr. Laverani*, etc., the blepharoplast occupies a position by the side of, or anterior to, the nucleus, and such forms might very properly be included under *Herpetomonas*. While this position of the blepharoplast is the usual one in cultural forms of trypanosomes, it is not always so. Thus, in some species, notably in the spirochete-like form of *Tr. avium*, it may lie posterior to the nucleus. This position, though rare, has also been observed in insect flagellates, namely *Tr. Tullochii* and *Herpetomonas algeriense*.

It is furthermore worthy of note, showing as it does the close relationship which exists between the cultural trypanosomes and the *Crithidia* and *Herpetomonas*, that all three types give rise to essentially the same gregarine forms. These are attached, whip foremost, to the epithelial cells of the intestinal wall, and are crowded together in several layers; or they form large rosettes with central flagella or agglomerations filling the lumen of the tube. The polymorphism noted by Léger in the case of *Crithidia* and *Herpetomonas*, and by Schaudinn for *Tr. noctuae*, is equally marked in cultures of blood trypanosomes.

According to Léger, the main difference between *Crithidia* and *Herpetomonas* is in the large size of the adult monadian form of the latter as compared with that of the former. The existence of a double flagellum, as claimed by Prowazek for the *Herpetomonas* of the house-fly, if confirmed, would afford a better generic distinction. Gray, it is true, has found a double flagellum on the *Herpetomonas* of the *Stomoxys*, but it is by no means certain that this condition does not represent an early stage of division.

In view of the cultural characteristics of these three types, we believe it justifiable to consider them as belonging to the one genus *Trypanosoma*. The terms *Crithidia* and *Herpetomonas*, as in the case of *Proteosoma*, etc., will undoubtedly persist, but not as representing distinct genera. With the recognition of the trypanosomal nature, it does not follow that all of these flagellates are capable of growing in the blood of higher animals. Parasitism in the living blood must be looked upon as the result of a previous adaptation to the more or less digested blood in the intestinal tract of sanguivora. From this standpoint it will be seen that the ancestral, and for that matter the normal, type is the one met with in the digestive tube. Hence, the cultural forms, instead of being regarded as degenerations or involution types, in reality represent the normal phase, whereas the blood forms are transitory modifications due to environmental conditions.

## METHODS EMPLOYED.

In order to study the flagellate infection of "wild" mosquitoes, the latter were captured in large numbers by means of Nocht's tubes. They were nearly all obtained from the thick undergrowth along the river bank in Ann Arbor; and, as soon as they were brought to the laboratory, they were placed in wire cages and allowed to feed on perfectly "clean" normal animals. By "clean" we mean animals which culturally and microscopically were shown to be free from trypanosomes and hemocytozoa. The mosquitoes were almost wholly of the genus *Culex*, and the most common species was *C. pipiens* or *pungens*. There were always a few *C. sylvestris* and *C. triseriatus;* the identification of these two we owe to Dr. L. O. Howard. The *Anopheles* were very scarce, only a few *A. maculipennis* and *A. punctipennis* being captured. For the percentage of infections see p. 242.

At first the insects were fed on young pigeons, but at times hawks, crows, rats, and guinea-pigs were made use of. In order to prevent the mosquitoes being killed, it is necessary, as a rule, to immobilize the animal. In the case of the pigeon this can be done by merely tying the wings against the body. Hawks were managed best by placing them on a Latapie holder, but the crows were with difficulty prevented from eating up the mosquitoes. Contrary to the general statement that these insects will not feed on rats, we have found that they do so, though perhaps not so freely as on pigeons. On placing the neck of the rat in the pillory of a Latapie holder, the mosquitoes will feed undisturbed. In general, the guinea-pig is the animal of choice, for the reason that it is readily attacked by the mosquitoes, and, since it does not attempt to destroy them, there is no necessity to secure it in any way. Although by no means necessary, it is advisable to trim off some of the hair or feathers on the exposed animal.

Usually the exposure to the mosquitoes took place during the early part of the evening, but the number of insects which then feed is relatively small. By allowing the guinea-pig to remain in the cage all night, a much larger number will feed, especially during the early hours of the morning. Each cage contained a hundred or more mosquitoes, but the majority of these would not feed. After removing the animal from the cage, the insects which fed were taken up in Nocht's tubes and set aside in a cool, shaded place. The wide end of the tube was covered with netting, while the trap end held a plug of cotton, moistened with water. It is not advisable to moisten it with sugar-water unless the insects are to be kept alive for many days.

The mosquitoes which thus fed on normal blood were kept from 30 to 70 hours, when they were examined for flagellates. The time for making the examination depended somewhat upon the temperature, for in very warm weather complete digestion took place in about two and a half days, whereas when it was cool three or more days were needed. The percentage of findings was rather low when the mosquitoes were examined early—that is, when the stomach was still distended with undigested blood. Evidently the flagellates had not multiplied sufficiently to permeate the entire contents, and hence could be easily overlooked. Similarly, when the examination was delayed until complete digestion had taken place, or when sugar-water had been fed, the flagellates were equally difficult to find, owing to the fact that the free-swimming or monadian forms, as designated by Léger, had passed into the resting or gregarine stage. The best results were always obtained when the examination was made toward the close of the digestion period—i. e., 40–60 hours after feeding.

In all of these experiments the mosquitoes were examined after a single feeding, and it is quite likely that the percentage of infections would have been even higher had they been fed two or three times so as to obtain an increased multiplication of the flagellates.

The mosquitoes were killed by inverting the tube over a filter paper on which was placed a drop of chloroform. This does not appreciably affect the flagellates, which remain alive and active in the stomach. After removing the thorax, wings, and legs, the digestive tube can be drawn out in the usual way, and the contents then forced out into sterile salt solution. Owing to the large number of examinations which had to be made, it was found that direct pressure on the dismembered body, by means of a sterile lance-shaped needle, gave about the same result, and this shorter procedure of getting at the stomach contents was the one chiefly used. By this means the fluid contents were expelled into a drop of sterile salt-citrate solution (a half per cent of each); the slide and cover-glass were previously flamed. The yellowish or whitish liquid was then examined with a No. 7 Leitz objective, and when necessary with an oil immersion lens.

When present, the flagellates are easily recognized by their more or less active motion. After a preliminary study of the organisms, the cover-glass was carefully drawn off, dried in the air, and stained by a modified Romanowsky method. When deemed advisable, the fluid remaining on the slide was taken up with a few drops of salt-citrate solution and either injected into birds or transferred to blood-agar tubes.

The flagellates present in the stomach contents were injected into 20 canaries, two mourning doves, and two blackbirds. The injections were made intrapleurally, and in no instance was it possible to observe the development of either trypanosomes or intracellular parasites in the blood of the birds thus inoculated. It may further be added that the pigeons, etc., after the mosquitoes fed on them were examined microscopically, and later cultures were made from their blood, but no evidence of infection was obtained.

The different cultures which were obtained were likewise injected into many animals such as sparrows, canaries, pigeons, doves, hawks, owls, crows, rats, and mice. Inasmuch as such cultures were very rich in flagellates, the number injected was clearly many thousand times greater than would be introduced into an animal by the bite of a mosquito. The results were negative, as in the inoculations with the stomach contents. It may be that the failure to produce an infection was because of the use of non-susceptible animals. Some vertebrate may be capable of infection with the mosquito flagellates, but as yet nothing is known on that point.

One infection experiment is specially worth mentioning. Four sparrows which had been previously examined and found clean were injected with a second generation culture of Strain No. 85 (*Crithidia*). After eight days one of the sparrows showed halteridia, another showed halteridia and proteosoma, while the other two remained clean. Apparently, two out of four sparrows developed halteridia as the result of the injection and it will be noticed that the same percentage, three out of six, was obtained by the Sergents in their work on the owl halteridium. It is reasonably certain, however, in our experiment that neither the halteridium nor the proteosoma was derived from the injected material. We have repeatedly found sparrows which at first were apparently clean, but after being kept in the laboratory for a week or two developed a halteridian or proteosomal infection. Latent infection is a common occurrence in sparrows, and for that matter in other birds, and, as pointed out on page 231, this condition may account for the apparently successful infections obtained by Schaudinn and by the Sergents.

For the cultural work the blood agar was prepared in the usual way. Defibrinated rabbit's blood was employed, and this usually was added, in the proportion of one to two, to the melted agar, previously cooled to about 50°. The slanted tubes, when solidified, were inoculated, by means of a drawn-out tube pipette, with the citrate suspension of the stomach flagellates, after which they were capped and set aside at the room temperature.

The chief obstacle to the successful cultivation of the mosquito flagellates is the constant presence of bacteria in the intestinal canal of the insect. The bacteria, on account of their more rapid multiplication, may outgrow the flagellates from the very start, and thus render the medium unsuitable for the latter. This may be brought about by a change in the reaction, or by the production of directly injurious products, or by the abstraction of oxygen. The abundant growth of aerobic bacteria may, as is well known, lessen the oxygen contents of a liquid to the extent of producing an anaerobic condition, which we know would interfere with the growth of trypanosomes in the culture-tube. In the stomach of the insect for some reason (possibly the reaction) the bacteria do not develop readily, and hence the trypanosomes are able to multiply, at times, to a very marked extent.

As might be expected, the flora of the insect gut is subject to considerable variation, and when slow-growing yeasts or bacteria predominate, the chances of getting the trypanosomes to grow, at least for one or two generations, are favorable. The conditions are seemingly more favorable when only a single species of bacterium or yeast is present. Our most successful results were obtained with such "pure mixed" cultures. The isolation of the trypanosomes from the accompanying bacteria or yeasts offers some difficulty, but this we were able to overcome, as will be shown later on, by plating on blood agar.

Cultures were attempted from 89 mosquitoes, all of which contained trypanosomes, some even in very large numbers. Of this number only seven were positive, Strains 10, 11, 18, 29, 69, 85, and A. Strains A and 69 were crowded out by bacteria after two and three days respectively. Strains 18 and 85 were kept alive for 20 days, through two and five generations respectively. Strain 11 was carried in 30 days though four, and Strain 29 in 166 days through 24 generations. Strain 10, which was started on August 5, 1905, is now (April 1907) in its 87th sub-culture.

Of the two cultures which were kept up one, Strain 10, was *Crithidia fasciculata* and the other, Strain 29, was a *Herpetomonas, Tr. culicis*. The former was associated with a yeast and the latter with a minute bacillus. The growth was very rapid and extremely rich, so much so that in warm weather it was necessary to check it by keeping them in a cold room. These two cultures will be described in their proper place.

## CRITHIDIA FASCICULATA.

This flagellate is fairly common in the gut of ordinary "wild" mosquitoes of the genus *Culex*, but it is not the only organism that may be present. Thus, out of 882 mosquitoes examined in 1905, flagellate infection was met with in 126 (14.3 per cent). Of this number, 55 had apparently only *Crithidia* (6.2 per cent), while 52 had only *Herpetomonas* (6.0 per cent), and 19 had both parasites (2.1 per cent). During the summer of 1906 the percentage of infected mosquitoes was about a third of that found in the preceding year. Thus, of 746 mosquitoes 37 were infected (4.95 per cent). Of this number 20 had only *Crithidia*, 16 only *Herpetomonas*, and one had a double infection. It will be seen from this that infection with the former is slightly more common than with the latter. The relatively small number of infections obtained last year was probably due to the cutting-away of most of the undergrowth along the river.

The existence of a mixed infection with these two flagellates has not heretofore been recognized, and as a result, the two types, when occurring together, have been described as different stages of one and the same organism. This is clearly the case with the *Herpetomonas subulata* of Léger, for the small pear-shaped, truncated forms which he shows (Ref. 35, p. 614, Figs. 4 and 5) are *Crithidia*, while the longer ones (Figs, 1, 2, and 3) are true herpetomonads. Again, the *Crithidia minuta* which Léger, on account of the presence of the long form (Ref. 35, Fig. 6, p. 614; also Ref. 33, Fig. 3, p. 185) later placed in the genus *Herpetomonas*, is without doubt a true *Crithidia*, since it shows the short pear-shaped as well as the elongated, cylindrical form which belongs to this type. These two figures of Léger should be compared with Fig. 6, Plate 8. Our reason for this belief is based upon the results of the cultivation of the two types, for it will be shown that in cultures the *Crithidia* and *Herpetomonas* present entirely distinct forms and retain their individual characteristics through numerous sub-cultures. In cultures of *Crithidia* the typical long herpetomonad form, gradually tapering posteriorly, is never found, and similarly in cultures of the *Herpetomonas* there is an entire absence of the peculiar short crithidian type.

On account of the facts just stated we cannot agree with Lühe in his characterization of the genus *Crithidia*. According to Prowazek, the *Herpetomonas* of the house-fly has a double flagellum arising from a terminal blepharoplast, but this observation, as already pointed out, needs confirmation. The absence of the double flagellum, the position of the centrosome at some distance from the anterior end, and the presence of a feebly developed undulating membrane are regarded by Lühe as the most important generic features of *Crithidia.* These properties, however, are based presumably upon the long form of *H. subulata,* which is entirely different from that of true *Crithidia.* Hence, until it is definitely shown that the *Herpetomonas* of the house-fly always possesses a double flagellum, it will be well to retain this designation for similar forms found in other insects.

*The genus* Crithidia, *if it is to be retained, is characterized by peculiar, short, oval or pyriform bodies which are usually rounded or obtuse posteriorly, while the anterior end is truncated, or even slightly depressed, and bears a short, straight flagellum. In this short form the nucleus is near the posterior end, and the blepharoplast is by its side. Somewhat longer cylindrical forms, tapering very slightly, or rounded at each end, may be present, and these are provided with a long flagellum. In this form the nucleus is near the center, and the centrosome is usually about midway between the nucleus and the anterior end. Both forms may occur in rosettes with flagella directed centrally. The undulating membrane and the posterior diplosome, as seen in the* Herpetomonas, *are absent.*

Some variation in the form and size of the *Crithidia* is met with in the body of the mosquito. This must obviously be the case in view of the changes in the composition and concentration of the intestinal contents. It will be sufficient to point out the great difference in the rate of digestion of the blood in the stomachs of mosquitoes according as they are kept at a high or a low temperature. Furthermore, the ingestion of plain water or of sugar-water is an additional factor in bringing about greater or less alteration in form and size. In the cultures on blood agar these differences are readily observed, as will be presently shown. The environmental conditions are essentially the determining cause of the two types of Léger—the free or monadian, and the fixed or gregarine-like, resting stage.

The possibility of the existence of several species of *Crithidia* is obvious, and it is likely that their differentiation, as in the case of bird trypanosomes, can be effected more readily by the cultivation method than by mere morphological study. In the course of this investigation we have grown five strains of *Crithidia*—Nos. 10, 11, 69, 85 and A; but unfortunately all but the first were soon lost. It was noticeable, however, that Strain No. 85 differed in some respects from the others, but this variation might have been due to changes in the medium caused by the different kinds of bacteria present. What has been said here with reference to the existence of several species of *Crithidia* is also true for the *Herpetomonas*. The question is worth investigating in localities where flagellate infection of mosquitoes is very common. Taken as whole, the *Crithidia* which we have studied, in mosquitoes and in cultures, agree so well with the description of *C. fasciculata* which Léger found in *Anopheles* that we have identified all of our forms with this species.

### MOSQUITO FORMS.

The flagellates may be present in the stomach contents in varying numbers. In some instances but one or two may be found on the slide, while in others they are exceedingly numerous. They may be single or in large masses which fill the field of a No. 7 objective. For convenience the *Crithidia* will be divided into the short and the long forms.

As seen in the living condition the *Crithidia* usually appear as short, thick, oblong cells. They are more or less actively motile and travel about with the whip foremost. The body of the smaller forms is about 3–4 μ long and 2 μ wide, while the larger ones measure 6–8 μ in length by about 3 μ in width. The contents are colorless and apparently homogeneous.

The short forms are often found grouped in rosettes with the whips inside. These groups may consist of large numbers of cells, just as in the case of the rosettes of *Tr. Lewisi*, *Tr. avium*, etc. The short form, furthermore, may become attached by means of the flagellum to the intestinal wall, more especially to the lower end of the Malpighian tubules, and as a result the entire wall may be covered with radiating masses consisting of countless numbers of parasites. A similar arrangement of the cells is to be seen about minute globules of air (aerotropism), and this behavior so characteristic of cultural trypanosomes will be discussed later on.

The most important features are brought out in the stained preparations. The thick wide cells may be said to be pear- or cigarshaped (Plate 7) and have been compared by Léger to barley grains. The posterior end is either round or slightly pointed. The body of the cell may, or may not, taper toward the anterior end, which is either square or slightly concave. Round forms, from 4 to 6 $\mu$ in diameter, may also be present. The relatively large round nucleus lies near the posterior end in the short forms and at about the middle in the longer ones; close to the nucleus in front or on the side is the centrosome. From the achromic zone surrounding the latter, the flagellum can be seen to pass forward and out *at the middle* of the anterior end. The free portion of the whip is short and straight, or but slightly bent. The flagellum is more difficult to stain than in the case of other trypanosomes, and hence the cells will often appear to be devoid of whips. A colorless channel, marking the site of the flagellum, can be seen in such specimens, extending from the blepharoplast to the anterior end (Figs. 1 and 2, Plate 8). In the stained preparations the body of the cells measures from 3 to 8 $\mu$ in length and from 1.5 to 3 $\mu$ in width. As the free whip is from 2 to 5 $\mu$ long, the total length of the cell may be put down at from 5 to 14 $\mu$. These measurements, it may be said, have been made on preparations made from mosquitoes which contained no herpetomonad forms. The absence of very long forms should be noted.

### CULTURAL FORMS.

As mentioned on p. 241, one of the crithidian strains which were isolated from mosquitoes (No. 10) has been cultivated now for 20 months, and during that time has passed through 87 generations or sub-cultures. For more than a year it has been grown as a strictly pure culture.

On the ordinary blood-agar medium it grows even more rapidly than any of our bird trypanosomes, so much so that during the warm weather it is necessary to restrain the growth by placing the tubes in a cool room. Without this precaution it would be necessary to transplant the culture every three or four days. The growth is extremely rich, and glairy patches or colonies are readily made out. Obviously, depending on the changing conditions of the medium and temperature, some variation in form and size will occur, but on the whole the cultural features of our strain have been maintained unimpaired during these many months.[1]

It is important to note that these cultural forms are very often of the same size and shape as are those found in the mosquito. This fact will be readily seen on comparison of the Plates 7, 8, and 9. As in the gut, so in the tube we can distinguish between a very short and a moderately long form. The short thick cells (body 4 to 8 μ long and 2 to 3 μ long) with truncated anterior are identical with like forms seen *in vivo*, the gregarine forms of Léger. On the whole, the conditions in the test-tube, notably the abundant food-supply and absence of bacteria and other inhibiting influences, are more favorable than in the insect, and there is a greater tendency to develop the long or monadian form. This is entirely different from that of the *Herpetomonas*, and, as will be seen, it is much shorter than the latter. The body of the long form measures from 8 to 15 μ in length and from 1.5 to 2 μ in width. The free flagellum may be as long as the body, and hence the total length of the cells may range from 13 to 26 μ. The width of some of the forms, especially just before division, may reach 4.5 μ. These long cultural forms are represented by similiar ones (10–15 μ in length), resembling *Herpetomonas*, which are found at times in the mosquito. The cylindrical or cigar-shape, the obtuse posterior end, and the very short taper to the anterior end of body, together with the absence of the posterior diplosome, makes it possible to distinguish the elongated *Crithidia* from the true *Herpetomonas*. (See p. 254.)

It is important to note that the long cultural forms, at times, show a marked constriction near the posterior end (Figs. 4 and 5, Plate 9). Although we have not ascertained the real significance of this constriction, we are inclined to consider it as a process preparatory to actual division. This feature corresponds, in a remarkable way, with the constriction of the oökinetes of *Tr. noctuae* as described by Schaudinn (see his Figs. 1, 2, and 3).

While this fact, together with the rounded posterior end, goes to establish a certain identity between *Tr. noctuae* and *Crithidia*, it may be well to point out that the former does not agree in all respects with the latter. This is especially true of the anterior end, which in *Tr. noctuae* is prolonged along the base of the flagellum and clearly shows an undulating membrane. These two characteristics, and the presence of posterior diplosomes, agree with the structure of *Herpetomonas*. It would seem therefore as if Schaudinn's *Tr. noctuae* was a composite of these two flagellate forms.

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[1] At the present time the vegetative power of this organism is such that it can be grown on ordinary inclined agar without the addition of blood. In several attempts to secure "blood" forms, by resorting to cultivation in collodium sacs in rats, no growths were obtained.

Rosette formation is a common feature of the cultures and corresponds to the characteristic grouping met with in insects. The smaller groups of from two to 10 cells usually consist of short thick ovals or spherical bodies which, as in the case of the rosettes of *Tr. avium*, *Tr. Lewisi*, etc., show little or no motion. At times the rosettes consist of many hundreds of individuals, which may be partly or wholly of the elongated type, and in such cases the latter show an active swaying motion. The flagella are directed centrally as in the case of all multiplication rosettes.

The rosettes are easily distinguished from ordinary agglutinations. The latter result in irregular masses, consisting at times of numerous cells, all of which have the flagella on the outside. The beginning of agglutination may be seen when two cells become adherent by their posterior ends; or side to side with the flagella extending in opposite directions.

Another very important feature which seemingly is shared by all cultural trypanosomes, but not by the blood forms, is the phenomenon of *aerotropism*. Thus, if a minute bubble of air is introduced under the cover-glass, the *Crithidia* will range themselves in compact rows, two to five deep, around the globule. Every cell has its whip directed toward the air-bubble. This behavior of *Crithidia*, and of cultural trypanosomes in general, to air corresponds, as will be seen, to the so-called gregarine or resting stage observed in the insect. In the latter the flagellates are attached in the same manner to the epithelial cells, and it is reasonable to believe that this so-called resting stage is in reality an aerotropic manifestation due to the alteration and absorption of the stomach contents. Certain it is that this resting form when detached from the epithelium, by pressure or currents of liquid, is at once able to move about quite freely.

The cultural fluid, in addition to the rosettes, shows many free, actively moving, darting forms. These may travel through the field with great rapidity and in nearly straight lines. Most of the single forms, however, for some reason become attached by means of their flagella to the glass surface and are unable to do more than sway or wriggle about the attached point.

The length of the flagella varies with that of the cell, and in general is about as long as the body proper. In the small forms the whip is short and stiff, while in the elongated type it is long and flexible. In the former it is not unusual to see the flagellum retracted to a short stiff bristle as a result of the passage of the blepharoplast from the anterior end of the cell to the side of the nucleus which lies at or near the further end. (See Figs. 1, 2, and 3, Plate 9.)

The larger *Crithidia* show a distinct yellowish green color, which may be due to staining with blood pigment. The contents of the cell appear homogeneous, or show at most a very fine granulation. In heavily stained preparations deeply colored granules can be seen filling the cell (Plates 8 and 9). This stippling is at times difficult to bring out. In Strain 85 the cultural cells were totally colorless and showed very minute but clear, distinct globules.

The presence of an undulating membrane has not been satisfactorily established. The short, oblong forms, on account of the square anterior end and median insertion of the flagellum, are clearly devoid of an undulating membrane. In the very longest forms the anterior end is not carried along the flagellum, as in the case of *Herpetomonas* and most trypanosomes, but is seen to terminate more or less abruptly. Such long forms, when examined in the living condition, especially when the cell has become sluggish, give evidence of the presence of an undulating membrane at the base of the flagellum. The periplast is apparently carried beyond the obtuse or truncated end of the cell as a short triangular membrane which shows a *distinct wave motion.* With the retraction of the flagellum, as in the short form, all evidence of this structure disappears. In the stained preparations it has not been possible to demonstrate this rudimentary membrane, although indications of it are to be seen in Fig. 6, Plate 8.

The presence of an undulating membrane, imperfect as it is, definitely places the *Crithidia* among the *trypanosomes*, and this position is further indicated by the obvious analogies which this type presents in cultures to those of the latter.

The very small rounded forms, especially when the flagellum does not stain, resemble very much, as Léger first pointed out, the *Piroplasma*. The resemblance to the peculiar parasite of kala-azar, *Piroplasma donovani*, is even more marked. The development of the latter into flagellate forms in the test-tube (Rogers), and in the bedbug (Patton) suggested to the former a relationship to *Herpetomonas*, while the latter is of the opinion that this parasite is a *Crithidia*.

Divisional forms, of course, are very common in the cultures, and hence such material presents the very best opportunity for the study of the life-history of the parasite. The forms met with are the same as those seen in the mosquito. The short, wide ovals divide longitudinally into two equal halves, each of which shows the typical square anterior end (Fig. 3, Plate 8).

As mentioned on p. 239, the inoculations of birds and mammals with large amounts of the flagellates, obtained from tube and flask cultures, failed to produce an infection. Hence, so far as we know, the *Crithidia* is a parasite peculiar to the mosquito, and perhaps to other insects.

### TRYPANOSOMA (HERPETOMONAS) CULICIS, N. SP.

It has already been shown that the ordinary "wild" mosquitoes of the genus *Culex* may harbor either *Crithidia* or *Herpetomonas*, or both kinds of parasites at the same time. From the figures given on p. 242 it will be seen that the infection with *Herpetomonas* is nearly as common as that with *Crithidia*.

The *Herpetomonas* which we have studied resembles in general the long monadian form of *H. subulata* which Léger found in *Tabanus glaucopis*, and at first we were inclined to identify it with the latter. Léger's form, however, is distinctly longer, the body on an average being 30 μ long by 1.5–2.0 μ wide, the flagellum being 20–25 μ long. Moreover, as pointed out on p. 242, the description of this species undoubtedly includes a true *Crithidia*. The *Herpetomonas algeriense* which the Sergents described, shortly after the appearance of our preliminary note, was found in 13 specimens of *Culex pipiens* and in one of *Stegomyia fasciata*, and was characterized as having the centrosome in the motile forms, posterior to the nucleus, and also by a rounded resting form having very long flagellum. The unnamed type which they found in the digestive tube of a larva of *Anopheles maculipennis* resembles our form more closely than does the preceding. It appears that, notwithstanding its frequent occurrence, this mosquito flagellate has not been heretofore recognized, or at all events, it has not been differentiated from the accompanying *Crithidia*. We therefore consider it as a new species and propose to designate it as *Trypanosoma culicis*.

While this organism has been found only in the several *Culices* examined by us, it is quite probable that it will be recognized in other mosquitoes, including *Anopheles* and *Stegomyia*. It is hardly necessary to add that a further investigation of the mosquito flagellates is needed in order to pass upon the question of the multiplicity of species both of *Crithidia* and *Herpetomonas*. The wide geographic distribution of these two types is seen in the fact that Patton has found both of them to be very common in the *Culex fatigans* at Madras. The probable presence of the two types or genera in other insects is evident from Léger's work on the Tabanides.

According to the recent studies of Prowazek, the herpetomonads of the common house-fly and of the meat-fly (*H. muscae domesticae* and *H. sarcophagae*) possess a double flagellum which terminates in a minute diplosome, and this, in turn, connects with a single large blepharoplast by means of two rods or rhizoplasts. As pointed out, if these observations are confirmed, it will be necessary to restrict the term *Herpetomonas* to those organisms having the same flagellar arrangement as in the type species *H. muscae domesticae*. It seems to us, however, somewhat premature to insist, as Lühe has done, upon the limitation of this genus to these two double flagellar forms. The herpetomonadian forms have been noted in diverse insects, but in none has this flagellar structure been seen. Such an arrangement is certainly absent in the herpetomonadian forms which we have studied, namely *Tr. culicis* and *Tr. Grayi*.

On the other hand, there are reasons for believing that the double flagellar arrangement described by Prowazek represents an early divisional stage. Such undoubtedly is the case with the forms described in the *Stomoxys* by Gray. It is to be remembered that the first evidence of division is usually seen in the enlargement of the blepharoplast, and then in the formation of a new flagellum (see

Fig. 3, Plate 11). As a matter of fact, in rapid multiplication, such as is seen in the segmenting forms of *Tr. Lewisi*, the cultural forms of *Tr. Laverani* (Ref. 46, Fig. 6, Plate 6), etc., the new whip appears before the blepharoplast shows any appreciable sign of division. The existence of a double flagellum would effectually remove the flagellates of the house-fly from the trypanosomes. The *Herpetomonas* of the mosquito, which it will be seen is a true "cultural" trypanosome, shows considerable similarity to *H. muscae*.

### MOSQUITO FORMS.

What has been said on p. 244 with reference to the variation in the form and size of *Crithidia*, and the possible existence of several species, holds equally true for the *Herpetomonas*. The conditions which obtain in any two insects are not necessarily alike, and, for that reason, considerable difference in size and form may be expected without recourse to the assumption that very long forms represent one species and very short ones another. The variation in size is an important argument in support of the view that the intestinal flagellates are true cultural types.

In the living condition the *Herpetomonas* is very active and moves about rapidly; so much so that it is often difficult to follow it from field to field. It moves with the long flagellum foremost in exactly the same manner as do the cultural trypanosomes, *Tr. Lewisi*, *Tr. avium*, etc. The contents of the cell appear colorless and homogeneous.

On account of the rapid motion the form is at times difficult to make out, but in cells which have slowed down, and especially in stained preparations, it will be seen that the cell is a narrow spindle tapering gradually toward each end. The presence of an undulating membrane is clearly indicated in the longer forms.

The herpetomonad forms seen in the living preparations vary considerably in length. In a few mosquitoes very long forms, the body measuring from 30 to 35 $\mu$, have been observed. In these the flagellates were all of this size, and, had the cultivation attempts been successful, it would have been possible, perhaps, to determine whether this long form was a distinct species. In the majority of the mosquitoes the herpetomonad forms were from 12 to 20 μ long. The width of the living cell is more uniform and varies from 0.5 to 1.0 μ. Hence, the appearance of the long, slender form is not unlike that of the spirochete-like stage of *Tr. avium.* It is a mistake, however, to compare either of these types with the true spirochetes, inasmuch as there is an entire absence of the spiral winding so characteristic of the latter and, moreover, on staining, there is brought out the usual trypanosomal structure which is wholly lacking in the real spirochetes (*Sp. Obermeieri*, *Sp. Duttoni*, etc.).

The presence of these very long, slender forms in the mosquito, however, is very strongly suggestive of Schaudinn's *Spirochaeta Ziemanni.* It will be remembered that Schaudinn himself practically acknowledged that this organism was not a true spirochete, since it had the ordinary trypanosomal structure. In view of this similarity, it may well be asked whether or not some of the forms of this *Herpetomonas* correspond with the flagellate stage of *Hemoproteus Ziemanni* as described by Schaudinn. Certain it is that the long, slender forms correspond to the size (25 by 1.0 μ) given by the Sergents for the flagellate stage of *H. Ziemanni.* On the other hand, it should be noted that Schaudinn's Fig. 17 shows a long undulating membrane, due to the position of the blepharoplast which is posterior to the nucleus, as in the cultural form of *Tr. avium.* For the cultivation of spiochetes from owls see p. 259.

In addition to the long and short forms mentioned, various stages of involution are met with as in the case of ordinary cultures. The more or less spherical forms with long flagella, kite-shaped bodies and spindles, rounding up posteriorly, correspond to the resting stage of Léger.

Divisional forms are often seen in the living preparations. The dividing cells are shorter and wider than the ordinary free-swimming forms. The division is longitudinal and is fairly equal. Multiplication rosettes with centrally directed whips are occasionally found, but they are by no means so common as in the culture tube. Agglutination groups or rosettes have also been observed, and it is important to note that in these groups the flagella are directed outward as in the case of *Sp. Ziemanni.*

The herpetomonad forms found in the stained preparations made from mosquitoes vary greatly in length, from 10 to 45 μ. They may for convenience, if for no other reason, be divided into four types: (1) long; (2) medium; (3) short; and (4) wide.

1. In the long spindles the posterior two-thirds of the body gradually tapers to a fine point, while the anterior third is drawn out along the base of the flagellum so that it is difficult to tell just where it ends (Fig. 2, Plate 10). The length of the body in these long forms varies from 25 to 35 μ, and the greatest width is from 1.0 to 1.5 μ. The free whip measures from 5 to 10 μ, so that the total length ranges from 30 to 45 μ. *With respect to size, as pointed out above, this type corresponds to the forms which the Sergents identified with the flagellate stage of* H. Ziemanni. (See p. 231.)

2. The cells of medium size,[1] those from 15 to 25 μ in length (not including the whip), likewise taper toward both ends. The width may be a trifle greater than that given for the preceding type—that is, from 1.5 to 2.5 μ (Figs. 1, 2, 3, Plate 10). *This type corresponds in size to the indifferent forms of* Tr. noctuae *as given by the Sergents.*

3. In the short form the body measures from 7 to 15 μ, and the whip is from 3 to 8 μ long. The width is about the same as that of the preceding. These short forms also show the characteristic tapering ends, though not to the same extent as in the long types (Fig. 6, Plate 10). *They may be compared with the male forms of* Tr. noctuae *as given by the Sergents* (p. 227).

4. In addition to the three types given above, which are by far the most common, there is another present, in mosquitoes and in cultures, though it is much less frequent. This is a wide, fairly long form in which the characteristic tapering is less pronounced (Figs. 5 and 6, Plate 12). The body measures about 20 μ in length and about 2.5–3 μ in width. The free whip is short, about 5–8 μ. This type stains a deeper blue than any of the above, and the contents show granules as well as colorless globules. *This form may be identified with the female form of* Tr. noctuae *as described by the Sergents* (see p. 227). It recalls the broad or "female" type of *Tr. Lewisi* as met with in the blood of rats, and may well be compared with the broad or "female" type of *Tr. Grayi* (Ref. 45, Plate 15).

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[1] The long and median narrow forms described above are suggestive of the slender or "male" type of *Tr. Grayi*, as will be seen on comparison of Figs. 1 and 2, Plate 10, with Figs. 1 and 2, Plate 15 (Ref. 45).

Inasmuch as all four of these types have been studied in the cultures of *Tr. culicis* as well as in the mosquito, there is every reason to regard them as belonging to one species. Similar variation in form and size, it may be added, is common among other cultural trypanosomes, for example, *Tr. avium.*

The nucleus in the narrow forms is compact and cylindrical, and as wide as the cell (compare *Tr. Grayi,* Ref. 45, Figs. 1, 2, and Plate 15). In the "female" type two nuclei may be present without there being any evidence of multiplication (Fig. 5, Plate 12). Similar polynuclear forms have been described in the cultural forms of *Tr. Grayi, Tr. Lewisi,* etc.

The blepharoplast is round or oval and measures 0.5–0.7 μ. It lies either immediately in front of the nucleus or about midway between the latter and the anterior end. We have never seen it lie posterior to the nucleus as in *H. algeriense.* Starting from the blepharoplast, a single flagellum passes forward along the side of body and is finally prolonged as the free whip. In the long forms the evidence of the presence of an undulating membrane is unmistakable (Fig. 2, Plate 10).

By far the most characteristic feature is the presence of a *posterior diplosome* or *acrosome* similar to that which we first described in the cultural form of *Tr. Laverani* (Ref. 46, p. 290, Plate 6), and in *Tr. Grayi* (Ref. 45, p. 407, Plate 16). This body is present in all four types as well as in the dividing forms. It appears to be a definite, constant structure, as much so as the nucleus or the blepharoplast. The presence of this diplosome has not been observed in *Crithidia.*

As a rule, the diplosome stains less readily than does the nucleus, and this behavior is probably due to the protecting action of the surrounding matter. In the very thin, or in crushed, cells this structure is as sharply defined as the blepharoplast (Fig. 6, Plate 11). The diplosome is usually rod-shaped, about 0.4 by 1.6 μ, with a more or less evident median constriction, in which case it presents the appearance of a diplococcus or diplo-bacillus (Fig. 1, Plate 10). It is nearly as large as the blepharoplast.

The diplosome apparently divides before any divisional change is seen in the nucleus or blepharoplast (Figs. 4 and 5, Plate 10). As the result of such division two distinct rods or two diplococcus-like bodies form. We have never seen more than two diplosomes in a cell, although special search was made. A suggestion of three such bodies may be seen in Fig. 5, Plate 10. In the first three types the diplosome is always posterior to the nucleus; in the fourth or "female" type it may lie in the anterior part of the cell.

A somewhat similar structure, derived from the blepharoplast, has been described by Schaudinn in connection with the development of the oökinete into the female and male forms of *Tr. noctuae* (see his Figs. 2 and 4). In that case the blepharoplast is said to give rise by three consecutive divisions to eight small, compact nuclei, each of which in turn divides, forming a smaller nuclear body. In the female cell, these eight pairs of nuclear bodies disappear, whereas in the male oökinete they become the nuclei and blepharoplasts of eight male trypanosomes. In his study of the *Herpetomonas*, Prowazek has likewise noted a posterior diplosome which is apparently in relation to the blepharoplast.

The diplosome which we have described is present in all forms of *Tr. culicis*, as found in the mosquito as well as in those grown in the culture-tube. The similar bodies of Schaudinn, it should be said were noted only in the oökinete. Just what the significance of the diplosome may be it is impossible to state, but our observations go to show that it is a distinct morphological structure and is not directly derived from the nucleus or blepharoplast. As pointed out above, the diplosome presents the earliest evidence of a coming division of the cell. As the latter divides, each half is equipped with a diplosome (Figs. 2 and 6, Plate 11), as in the case of *Tr. Laverani.*

The direct division of the long, slender forms of *Tr. culicis* have not been observed. They apparently shorten and widen just before division takes place. At all events, the common dividing form, as found in the mosquito and in the culture, is an oval body about 3 μ wide and 8–10 μ long. The acrosome divides first; then the blepharoplast, which lies just in front of the nucleus, divides and gives rise to a new flagellum, after which the nucleus divides (Fig. 3, Plate 11). The division is equal and longitudinal, and hence the resulting two cells are of about the same size about 8–10 μ long and 1.0 μ wide (Fig. 6, Plate 11).

The female type apparently divides longitudinally without becoming round or oval (Fig. 6, Plate 12). The two large nuclei which result from division separate, and one lies in the anterior and the other in the posterior part of the cell. Hence, one of the cells resulting from the division will have its nucleus at or very near the posterior end. In this dividing form the diplosomes lie either between or in front of the nuclei (Fig. 5, Plate 12), as in the case of *Tr. Grayi.*

*Involutions.*—In some mosquitoes the *Herpetomonas* loses its typical form, rounds up posteriorly, and becomes first club-shaped, and finally more or less spherical. This change likewise occurs in the culture-tube, and the resulting bodies correspond to the round form seen in cultures of *Tr. Lewisi*, *Tr. avium*, etc. Such bodies, measuring about 5 μ in diameter or 5 by 8 μ, represent in part the resting or gregarine form of Léger and others, and should probably be interpreted as degenerations or involutions, since the plasma in these altered cells does not stain as well as in normal cells. In such cells, owing to the feebly stained protoplasm, the nucleus, blepharoplast, and diplosome stand out very prominently (Figs. 3, and 4, Plate 12). As opposed to the view that these are degenerations is the fact that these forms show the various division changes. Thus, one may find all stages from the cell with a single nucleus, blepharoplast, diplosome, and whip to one in which each of these structures is doubled.

## CULTURAL FORMS.

Of the seven cultures of mosquito flagellates only one (Strain No. 29) gave a good growth of *Herpetomonas* which was successfully carried through 24 generations or sub-cultures in the course of five months, from August 6, 1905, to January 12, 1906. Another, Strain No. 18, went through two passages, but was then lost on account of overgrowth by the accompanying bacteria. The cultures made from Mosquitoes 10 and 11 which had a double infection showed some herpetomonad as well as crithidian forms (in the first generation), but for some reason the former disappeared on transplantation, and the second and subsequent generations showed only *Crithidia.* The disappearance of *Herpetomonas* from these cultures indicates some unfavorable action either of the particular kind of bacteria which were present or of the *Crithidia.*

Strain No. 29 was associated with a minute diplo-bacillus which seemingly exerted no injurious action as long as the cultures were transplanted regularly, once or twice a week. When the transplantation was delayed for a longer interval, most of the trypanosomes, as is usually the case, became degenerated, and hence the sub-cultures then made were poor in flagellates and relatively rich in bacteria. Otherwise there was no reason why this culture could not have been kept up almost indefinitely as in the case of *Crithidia.*

The isolation of a strictly pure culture was more difficult than with *Crithidia* on account of the adherence of the minute bacillus to the sticky surface of the flagellate. Several times, however, almost pure cultures were obtained from colonies on blood-agar plates, and, without doubt, this method on further replating would have given a strictly pure growth. Unfortunately, just at this time the culture was lost through an oversight in not making regular transplants. The streak cultures on rabbit blood agar, in a Petri dish, gave moist, glairy colonies of almost pure trypanosomes. The colonies varied in size from mere points up to that of a large pin-head.

The forms met with in the culture-tube, it may be said, correspond in every respect to those observed in the mosquitoes. The four types found *in vivo* are also present *in vitro*, and in size and form the latter are not to be distinguished from the former. This fact accords with the observations on the crithidian cultures, and confirms the view that the flagellates of mosquitoes and other insects represent cultural forms.

Nothing is known as yet regarding the forms which these flagellates would assume if they were able to grow in the blood current. It is probable, however, that they would give rise to large typical trypanosomes corresponding to the blood forms of *Tr. avium*, *Tr. Lewisi*, etc. It is evident, therefore, that the cultural forms, instead of being degenerations and involutions as suggested by some, really represent the ancestral primitive type from which the peculiar blood forms have evolved as a result of adaptation to the living fluids of the body. From the cultural stand-point the "drawn" blood does not correspond to the living blood, by which term we may designate that which is circulating in the body.

The cultures of *Tr. culicis* show small forms, the body of which measures but 6 or 7 μ. These may have a tapering posterior end, or one which is blunt and rounded. They are readily distinguished from the wider and truncated *Crithidia*. Small, rounded or oval forms, 4 or 5 μ in diameter, are also present, and such usually have a very long flagellum, 12–16 μ in length. The most common form is that of the typical *Herpetomonas* with slender, tapering posterior end. These are from about 12 to 15 μ in length, and from 1 to 1.5 μ in width. Occasionally longer forms up to 35–40 μ, and the "female" forms already mentioned, are met with (Plate 12).

Rosettes, with the flagella directed centrally, are present in the cultures, but not as commonly as in the case of *Crithidia*. Agglutination of two or more cells is a common condition. The two cells may be attached either by their posterior ends or lengthwise, side by side, with the flagella pointing in opposite directions. When agglutinated in large masses the cells are also grouped with the flagella on the periphery. As with other flagellates the presence of soft débris or fat globules favors the formation of large agglutinations.

Aerotropism is as pronounced as in the case of *Crithidia* and other cultural trypanosomes. The long, slender herpetomonads arrange themselves around the globule in dense layers, side by side, with the flagella inward, and thus present a very striking object.

The slender forms travel rapidly, with the whip foremost, and while in motion appear as mere lines. The body is straight or slightly curved, and is rather stiff—a condition especially noticeable when the motion has decreased. The contents of the living cell have a greenish tint and homogeneous, but on careful examination very fine granules can be seen, especially in the posterior half. The presence of a slight, but distinct, undulating membrane can be observed in the slowly moving, or arrested long forms. Short, wide dividing forms (6–8 μ long by 4–5 μ wide) corresponding to those observed in the mosquito are fairly common (Plate 11).

Inoculations of the cultures were made into a variety of birds and mammals, as stated on p. 239, but in no case was it possible to secure an infection showing either trypanosomes or cytozoa. While it is possible that this flagellate has a blood stage in some vertebrate, there is as yet no evidence of that condition, and, for the present at least, the *Tr. culicis* must be regarded as a parasite peculiar to the mosquito.

## SPIROCHETES.

The so-called *Spirochaeta Ziemanni* which Schaudinn described in *Culex pipiens*, as the flagellate stage of *Haemoproteus Ziemanni*, is clearly a trypanosome and not a true spirochete. In view of this fact, it is of interest to note that Töpfer, according to Mühlens and Hartmann, has succeeded in cultivating a spirochete from the owl. Although Töpfer has not published as yet his results, we are assured by Dr. Schilling that the organism mentioned is a real spirochete, and, that being the case, it throws much light upon the exact nature of "*Spirochaeta*" *Ziemanni*, which Schaudinn himself practically acknowledged to be a trypanosome. Töpfer's observation would go to show the existence of a spirochete infection of owls similar to that known in chickens and geese, bats, etc. It is possible that mosquitoes feeding on owls, thus infected, would show true spirochetes in the digestive tube.[1]

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[1] Since the above was written one of us (Novy), through the very great courtesy of Dr. Töpfer, has been able to examine a preparation and a photogram of the spirochete which he has cultivated from the blood of an owl infected with *H. Ziemanni.* There is no question as to the spirochetal nature of the organism which Dr. Töpfer has isolated. It is an interesting and important fact that this organism presents in cultures not only typical spirochetes 10–15 $\mu$ in length, but also smaller forms consisting of but one or two turns. The latter correspond to the S-, or vibrio, forms of the cholera spirillum. The cultures also show minute round bodies which contain one or two chromatin granules and these bodies doubtless represent the resting stage of *Sp. Ziemanni* as described by Schaudinn. They may be compared with the well-known involution forms of the cholera vibrio, and their presence, together with the short forms mentioned above, affords good evidence of the bacterial nature of this spirochete.

Dr. Töpfer's organism in all probability corresponds to the *Spirochaeta Ziemanni* of Schaudinn. The long spirochetes, and the short forms representing the minute ones found by Schaudinn in the gut of mosquitoes (the result of the rapid multiplication of the indifferent form), and also the round or resting forms, are in accord with Schaudinn's description. It appears probable, therefore, that the *Spirochaeta Ziemanni* after all is a spirochete and not a trypanosome. But assuming that this identity is established it does not go to prove that the spirochete in question is a stage in the development of the intracellular parasite *H. Ziemanni.* On the contrary, it indicates the existence of a spirochetal disease of owls corresponding to that of geese and chickens. Indeed, Schaudinn himself noted that the owls, inoculated with suspensions of mosquitoes, showed an enormous increase of indifferent spirochetes very much like that seen in infections with *Sp. anserinum.* He considered this as the acute stage which was later followed by the appearance of the sexual forms of *H. Ziemanni.* It may be said, as a result of our own observations, than an infection with *Sp. Obermeieri* or *Sp. Duttoni* seems to exert a marked favoring action upon the multiplication of trypanosomes and probably of other protozoa, and hence the change from one type to the other, as noted by Schaudinn, is suggestive of a double infection. It will be seen, therefore, that the owls are subject to infection with at least four parasites, namely *H Ziemanni*, *Halteridium*, *Trypanosoma*, and *Spirochetes*. *This fact of a multiple infection in owls together with the normal presence of flagellates in mosquitoes goes to show the great difficulties under which Schau-*

In view of the frequent occurrence of spirochetes in the mouth, stomach, and intestines of higher animals, the presence of these organisms might be expected in the gut of insects. As a matter of fact, several instances of this kind are now known. The three pathogenic spirilla, *Sp. gallinarum*, *Sp. Obermeieri*, and *Sp. Duttoni*, have been found in their insect hosts. The Sergents in 1906, reported the finding of spirochetes in large numbers in a preparation made in 1901, from the digestive tube of a larva of *Anopheles maculipennis.* These measured 8–17 μ (average 13 μ), and showed from 1.5 to 4 weak, spiral turns. At times they were agglutinated. A very similiar, if not identical, organism, *Spirillum glossinae*, was described shortly afterward by Novy and Knapp. It was present in the stomach contents of two tsetse flies (*Glossina palpalis*). The single cell or short form with three to four turns measured 8 μ, while the double cells were about 15 μ long. Another instance of this kind is given by Wenyon,[1] who found spirochetes in lice which infested mice having *Sp. muris.* There was no sign of multiplication, and attempts at infecting mice by means of lice were unsuccessful.

In our own work with mosquitoes we have not been successful in finding real spirochetes, although special search was made for them. In the stained preparations made from several mosquitoes we did find spirochete-like forms but the presence of foreign particles made their nature rather uncertain. We learn through a personal communication from Captain Patton that true spirochetes are very common in the mosquitoes about Madras. It is evident, therefore, that in some localities the genuine spirochetes may be detected in mosquitoes, flies, etc., more readily than in others.

## BEHAVIOR OF MAMMALIAN TRYPANOSOMES IN MOSQUITOES.

As bearing upon the question of the multiplication of trypanosomes in the gut of insects, it may be of interest to present some results obtained with *Tr. Lewisi* and *Tr. Brucei.* Wild mosquitoes were allowed to bite infected rats and guinea-pigs, and after a variable

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*dinn worked while endeavoring to establish the life-cycle of* H. Ziemanni. Now that attention is called to the probable existence of a spirochetal disease of owls it is important that a special effort be made to detect the organism in their blood and to demonstrate the action of pure cultures upon clean birds, and their behavior in the gut of mosquitoes.

[1] *Jour. Hyg.*, 1906, 6, p. 583.

length of time the stomach contents were examined and injected into rats and mice. The combined contents of from two to ten mosquitoes were injected into each animal.

No multiplication of these trypanosomes was observed in the stomach even in mosquitoes which were re-fed. The number of trypanosomes ingested rapidly decreased, and dead forms were found after a few hours. In the Lewis mosquitoes, flagellates were found alive as late as 65 and even 114 hours, but these did not resemble either the blood or the cultural forms of *Tr. Lewisi*, and without doubt were ordinary herpetomonads. Unquestionable survivals were noted occasionally as late as 36 and even 48 hours. The *Tr. Brucei* died out more rapidly than the rat trypanosomes, but a few were seen to persist in some mosquitoes as late as 30 and even 36 hours.

Of 16 rats injected with the Lewis mosquitoes, six died from bacterial infection in from three to 11 days, and 10 were under observation for over a month. In none of these rats were trypanosomes detected. The injections were made one and one-half, three, six, nine, and twelve hours after the infective feed.

Of 10 mice inoculated with the Bruce mosquitoes, two developed an infection after an incubation period of eight days. One of these, which received the stomach contents of two mosquitoes, nine hours after feeding, died in 11 days; the other, which received the contents of five mosquitoes 12 hours after feeding, died in 10 days. Two mice which received stomach contents, 30 and 36 hours after feeding, died in four and five days respectively from bacterial infection. Of four rats inoculated with the stomach contents only one became infected after a period of incubation of nine days. This received the stomach contents of three mosquitoes, 14 hours after feeding, and died in 13 days. On looking over our laboratory notes we are unable to find any record of successful infection of mice with mosquitoes which had fed 24 to 36 hours before. Hence the statement to that effect, made in a previous paper, is erroneous. Positive results, it will be seen, were obtained at the end of nine, 12, and 14 hours, but not always. On account of variable conditions in different mosquitoes, the infection experiments do not always succeed in the stated intervals or even in shorter ones. Thus, we obtained failures at the end of six, 12 and 14 hours, as well as in 18, 24, 30, 36, and 42 hours.

It will be seen from the above that the conditions in the stomach of the mosquito are vastly more unfavorable than in the culture-tube. In our previous studies on *Tr. Brucei*[1] it was shown that the sluggish survivals in the culture-tube (after inoculation with fresh blood) were usually non-virulent after the fourth or fifth day, and that if infection did occur it was only after a long period of incubation. In the above mosquito experiments the survivals failed to infect after 14 hours. The failure to infect with *Tr. Lewisi* is probably due to the same cause. Apropos of these tests, it may be recalled that Bruce was unable to infect dogs by injecting the stomach contents of tsetse flies after an interval of more than half an hour from the time of the infective feed. The fact that the ingested trypanosomes lose their virulence so rapidly in the stomachs of insects indicates a loss of functional activity, especially the power of multiplication and hence such insects cannot play the part of an active host. In view of these unfavorable conditions, it is difficult to see how the mammalian trypanosomes which are no longer able to multiply, even when placed in susceptible animals, could have a life-cycle within such a host.

### TRYPANOSOMES OF TICKS.

The detection of trypanosomes by Weber, in the blood of some cattle suffering with Texas fever, and the subsequent finding by Schaudinn of like organisms in restained preparations of the intestinal contents of ticks, taken from infected cattle in Finland, led the latter to set up the working hypothesis that the development of the piroplasmes of cattle and dogs is similar to that of the halteridia. That is to say, Schaudinn was led to believe that the piroplasmes possessed a trypanosomal stage resembling that which he had described for the intracellular parasites of the owl.

In view of the position assumed by him, and its acceptance by some writers, it will be of interest to note that trypanosomes have been recently found at Madras in a dog-tick, *Rhipicephalus sanguineus*, by Captain S. R. Christophers, I. M. S. He found the organisms in only one specimen of the tick, and the slide containing these flagellates he very kindly placed at our disposal. The preparation, which was made from the lower end of the Malpighian tubule or portion of the rectum, showed a considerable number of well-stained trypanosomes. We propose to designate this flagellate as *Tr. Christophersi.* Another instance of flagellate infection of a tick is mentioned by Doflein, who quotes Leydig as finding them in *Ixodes testudinis.*

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[1] *Jour. Infect. Dis.*, 1904, 1, p. 17.

From the photographic reproductions given in Plate 13 it will be seen that the flagellates of the dog-tick are typical "cultural" trypanosomes. They are considerably larger than the *Crithidia* and *Herpetomonas* of mosquitoes, and are quite different from the trypanosomes in tsetse flies, as will be seen on comparison with the microphotographs of the latter.[1] They resemble somewhat the cultural forms of certain bird trypanosomes which we have very briefly described under Type 4 in a previous paper,[2] but this of course does not imply that they are derived from avian flagellates. It is more likely that they will be found to come from mammalian forms.

The *Tr. Christophersi* is characterized by a prominent undulating membrane which extends over about half the length of the body. The roundish nucleus lies at about the middle of the cell, and is nearly of the same width as the latter. The small blepharoplast, about 0.7 μ in diameter, is usually close to and on one side of the nucleus, but at times it is found to lie immediately in front. From the widest central portion the body tapers in both directions. Anteriorly it gradually merges with the flagellum, and posteriorly it terminates in a sharp, pointed end very much as in the case of *Tr. Lewisi.* Minute colorless globules are present, especially in the posterior half of the cell. Division is longitudinal and unequal.

The trypanosomes are fairly uniform in size and are all of the same type. The total length is from 30 to 45 μ. The body is usually about 25 μ long, but may vary from 20 to 35 μ. The width is from 2.5 to 4 μ. The length of the free flagellum is 8–12 μ.

The size of these tick flagellates is of itself sufficient to exclude any possibility of their being developmental stages of the *Piroplasma canis.* If there was any doubt on this point, it has definitely been set aside by the recent studies of Captain Christophers, from which it appears that he has succeeded in following out the complete life-history of this organism. His work demonstrates that in the life-cycle of this cytozoon there is no trypanosomal stage. There is therefore very little probability of a flagellate stage being found in the other piroplasmes, cattle, horse, etc. All of the known facts with reference to insect trypanosomes go to show that those present in ticks are either derived from like forms in the blood of animals on which they feed or that they are parasites peculiar to these insects.

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[1] *Jour. Infect. Dis.*, 1906, 3. Plates 15, 16, 17.

[2] *Ibid.*, 1905, 2, p. 292.

### RELATION OF THE INSECT FLAGELLATES TO THE BLOOD TRYPANOSOMES.

This subject has been discussed (p. 234), in part, in connection with the account of the various investigations on insect parasites. It has been shown that abundant caution must be used in the interpretation of such findings. A natural parasitism of the intestinal tract is clearly a widespread and common condition, and it must be conceded that up to the present time no incontestable evidence has been produced to show that the sanguicolous trypanosomes are able to grow and multiply in insects. The facts, such as they are, indicate that the biting insects merely transmit the surviving unaltered trypanosomes which they have taken up with the blood, and that such transmission can occur only during the few hours following the infective feed. The innocuousness of biting insects, such as *Glossina morsitans*, *Gl. palpalis*, rat lice, etc., after they have digested the infected blood, can be interpreted in only one way, namely, that the ingested trypanosomes have died out or are about to do so.

The conditions in the gut of insects are comparable with, but hardly as favorable as, those which are offered in the culture-tube. Knowing the difficulties encountered in the cultivation of *Tr. Brucei*, *Tr. gambiense*, etc., it will be readily understood that even greater ones must exist in the intestinal tract. Hence it is that surviving forms of these organisms are rarely found in the gut after 24 to 36 hours following their introduction with the blood. The more rapid the digestion and absorption of the food, the more rapidly will such organisms perish in the digestive tube.

In the case of *Tr. Lewisi*, *Tr. avium*, etc., which may be looked upon as nearly "saprophytic" on account of the extreme ease with which they can be grown in the test-tube, it is evident that they are more likely to take on the "cultural" form, and thus adapt themselves to the new conditions than are the truly pathogenic trypanosomes. For this reason it is conceivable that these trypanosomes may multiply in the gut of lice, mosquitoes, etc., but the proof is as yet wanting.

The only instance which we have of the multiplication of the blood trypanosomes in the gut of sanguivora is that afforded by the leeches. The large quantity of blood taken up, the anti-coagulative action of the secretions, the slow digestion, and the low temperature are factors which enable trypanosomes of the type mentioned above to multiply in this intestinal tract, and thus, so to speak, secure a foothold. Hence a leech, once infected, is capable of transmitting the infection to eels, fish, and frogs, etc., even after some weeks have elapsed from the time of the infective feed.

It has been repeatedly shown that the flagellates present in the intestines of insects and leeches present the characteristics of cultural forms of trypanosomes, and this fact is now definitely proven for *Crithidia* and *Herpetomonas*. These organisms do grow and thrive in the test-tube vastly better than in the mosquito, and the rich cultures thus obtained afford the best material for the study of "the parasite's life-history and biology." The *ex cathedra* condemnation by Woodcock of the cultivation method on the ground "that this is not a zoological method of research" will hardly be accepted by the true investigator who is not bound down by set forms and procedures. Scientific progress demands the application of new methods, and though "the trypanosomes are not bacteria," it is safe to say that the cultivation method as applied to these organisms will yield results which might be utilized to advantage by the morphologist.

Bearing in mind the fact that the blood trypanosomes when grown *in vitro* lose their original form and present cultural characteristics which ally them to the gut flagellates, it will be seen that the blood form in all probability represents the latest adaptation, to a new environment, of organisms which originally are derived from the intestine of insects. The adaptation to living matter is obviously the sequel of a previous existence on dead matter, and for that reason the intestinal or cultural forms instead of being involutions or degenerations, closely represent, as Brumpt first pointed out, the ancestral or primitive type of the sanguicolous trypanosomes.

### RELATION OF INSECT FLAGELLATES TO CYTOZOA.

A brief statement of this question, by way of a summary, will not be out of place in connection with the foregoing topic. The demonstration of at least two distinct flagellates, *Crithidia* and *Herpetomonas*, in all stages of ordinary mosquitoes, goes to show beyond any doubt that these forms bear no immediate phylogenetic relation to the intracellular parasites. The recognition of this fact more than ever invalidates the observations of Schaudinn on halteridium and leucocytozoon. It is imperative that his work should be repeated with the same species of bird (*Athene noctua*), the same species of mosquito (*Culex pipiens*), and if possible in the same locality, namely Rovigno, and that under experimental conditions which will leave no room for criticism. The several possible sources of error have already been pointed out (p. 230), and until that work is confirmed it is unwise to give it undue prominence and to base on it far-reaching conclusions as some zoologists have done.

We are firmly of the belief that the large typical trypanosome which is commonly present in owls corresponds closely to the large form or "female" of *Tr. avium* which we have described and illustrated in a previous paper.[1] The similarity will be evident on comparing our photographs with the one given by Nocht[2] and designated as the female form of *Spirochaeta Ziemanni.* Hence our further belief that the owl trypanosome is no more related to the *H. Ziemanni* than is *Tr. paddae* to the halteridium which often accompanies it. Our work on the "Bird Trypanosomes" has shown conclusively that the blood trypanosomes are definite species and are not stages in the life-history of cytozoa. In our next paper we expect to show beyond doubt that *H. Ziemanni*, in the blood, has a different development from that worked out by Schaudinn. Moreover, we have allowed "raised" mosquitoes to feed on hawks infected with *H. Ziemanni*, and have not been able to secure the "spirochetes" or other evidence of flagellate stages. It may be added, parenthetically, that we have not been able to detect flagellates in a large number of larvae which were examined for that purpose, and hence this may account for the negative results just mentioned. Had we allowed "wild" mosquitoes to feed on the infected hawks, we should have obtained, without doubt, a flagellate infection as did Schaudinn and the Sergents. The fact that the latter used "raised" mosquitoes does not exclude the presence of intestinal flagellates, since it has been shown that in the warm countries infection of larvae is a not uncommon occurrence. The present study shows that the ordinary "wild" mosquitoes do harbor herpetomonad forms which conform fully to the "spirochetes" which the Sergents considered identical with those described by Schaudinn (p. 253). The relation of Töpfer's owl spirochete to *H. Ziemanni* has been discussed on p. 259.

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[1] *Jour. Infect. Dis.*, 1905, 2, Plate 3.

[2] Kolle and Wassermann's *Handbuch*, Ergänzungsband, Erstes Heft, 1906. Plate 3, Fig. 3.

The position of *Trypanosoma noctuae* is essentially like that of *Spirochaeta Ziemanni.* Inasmuch as the halteridium infection of owls and hawks in Michigan is much less frequent than that with the leucocytozoon, we have not been able to make any feeding experiments with mosquitoes in this type of infection. The study of the natural mosquito parasites, however, shows the same possible source of error and leads to the conclusion that the so-called *Tr. noctuae*, instead of being a developmental form of the intracellular halteridium, is a common inhabitant of the gut of mosquitoes, and is probably a mixture of crithidian and herpetomonad forms.

It is certainly significant that, in spite of the stimulus which Schaudinn's observations have given to the examination for flagellate stages, no one has yet demonstrated such stages for any one of the intracellular parasites. Thus, Billet's supposition that the *Tr. inopinatum*, in frogs, was the flagellate stage of a *Drepanidium* has been effectually disproved by Brumpt. The guarded supposition of Koch that the trypanosome present in crocodiles is in relation either with the accompanying hemogregarine or with the tsetse-fly flagellates (*Tr. Grayi*) will hardly stand the test of actual experiment. Lastly, the work of Thiroux has clearly shown the absence of any genetic relation between *Halteridium* and *Tr. paddae*, and the recent studies of Christophers are equally conclusive as to the absence of a flagellate stage in the life-cycle of *Piroplasma canis.* The "flagellate-like" forms recently described by Kinoshita for this organism have very little that is suggestive of trypanosomes.

## ISOLATION OF TRYPANOSOMES FROM MIXED CULTURES.

F. G. NOVY AND R. E. KNAPP.

In general, it may be said that bacteria once introduced into a culture of trypanosomes tend to outgrow and check the development of the flagellates. It is probable that this injurious action is due largely to the production of poisonous products or to an alteration in the reaction of the medium. The antagonism between certain bacteria and these flagellates is deserving of attention. In exceptional instances, however, the bacteria exert little or no interference, and may even be apparently beneficial. While in the former instance the trypanosomes soon die out, in the latter the mixed culture can be kept, by frequent transplantation, almost indefinitely. We have, for example, maintained a mixed culture of *Tr. Mesnili* associated with a coccus for nearly a year. Similarly, *Crithidia fasciculata*, which was isolated from a mosquito along with a yeast, was cultivated in this mixed form for nearly six months. Likewise, the *Tr. culicis* was grown in mixed culture with a minute diplo-bacillus for nearly five months. In these instances the foreign organisms did not alter, or but very slowly, the color of the blood agar, and it would seem from other trials as if a rapid destruction of hemoglobin by the accompanying bacteria was an injurious factor.

The isolation of trypanosomes from the gut of insects in a pure condition is hardly to be expected, owing to presence of various bacteria and yeasts. There is a possibility, however, of obtaining a "pure, mixed" culture—that is, where the flagellate is associated with only one kind of foreign organism—and such cultures as seen from the above examples are more favorable for transplantation than when a number of different species are present. The need of strictly pure cultures is soon recognized when it is desired to study the pathogenic action of the trypanosomes.

We have attempted to secure a separation by three different methods. In the first method fairly good results were obtained by inoculating the culture medium some 2 or 3 cm. above the condensation fluid, and then keeping the tubes in an almost horizontal position.

This was done with the object of allowing the motile trypanosomes to travel away from the accompanying bacteria or yeasts, and thus reach the fluid at the bottom of the tube. Several times almost pure cultures were obtained in this way, especially in the case of the *Crithidia* which was associated with a large yeast. Fairly good results were also obtained with the *Herpetomonas*, but in that instance the minute bacillus was more readily carried by the sticky flagellates into the condensation fluid. In the presence of motile or slimy bacteria this method fails entirely.

In the second method the attempt was made to utilize the marked aerotropism of the trypanosomes. As has been pointed out, the cultural flagellates tend to gather in compact layers about minute bubbles of air. On removing such air-globules by means of a capillary it would seem as if almost pure transplants could be obtained. The method, however, was not tested sufficiently to show its practicability.

The method which gave perfectly satisfactory results was as follows: By means of a glass spatula, made by drawing out the end of a glass rod, a little of the mixed culture was spread in a series of streaks over six Petri dishes containing solidified blood agar. Ordinary agar may be used in the first three dishes, since the desired dilution is not attained until in the last three. The form of Petri plate known as the "Kriegsministeriums-Modell," made by Greiner and Friedrichs, is particularly adapted for this purpose, inasmuch as it can be effectively sealed by means of a wide rubber band. The sealed dishes were then set aside at room temperature for 10 or 12 days. The last plate or two of the series was found to contain isolated colonies of trypanosomes, which could then be transplanted in the usual usual way to the test-tube. By this means we were able to secure a pure culture of *Crithidia*, and were almost successful with *Herpetomonas*. Without doubt this method will be found useful in future studies of the flagellates found in the gut of insects and other sanguivora.

## SUMMARY.

The results and conclusions of this study can be recapitulated as follows:

1. Of the "wild" mosquitoes examined by us approximately 15 per cent in one year, and about 5 per cent in that following, were

found to be infected with flagellates belonging to two easily differentiated types, *Crithidia* and *Herpetomonas*. The percentage of infections is influenced largely by local and seasonal conditions.

2. By the cultivation method it has been possible to isolate two of these flagellates, namely *Crithidia fasciculata* and *Tr. culicis;* and it has been shown that the plate method is applicable for the separation of trypanosomes from the accompanying bacteria and yeasts.

3. The organisms obtained *in vitro* correspond to those observed *in vivo*, and hence the intestinal types represent true cultural forms.

4. The two types are common in other insects, and, instead of being classed as distinct genera, they should be placed under the trypanosomes.

5. The mosquito flagellates are not stages of intracellular organisms, but are probably parasites peculiar to the insects.

6. The inoculation of available experimental animals with the *Crithidia* and *Herpetomonas* has given negative results.

7. The *Tr. noctuae*, as described by Schaudinn, presents the mixed characteristics of *Crithidia* and *Herpetomonas*, while that of the Sergents agrees with the *Herpetomonas;* as such, it does not represent a stage of the *Halteridium*.

8. The cultivation by Töpfer of a spirochete from the blood of an owl, presumably identical with Schaudinn's *Sp. Ziemanni*, indicates the existence of a new spirillosis. The forms which the Sergents identified with *Sp. Ziemanni* were probably not spirochetes but trypanosomes, more especially the long form of a *Herpetomonas*. The conclusion seems justified that neither the flagellates and spirochetes found in mosquitoes nor the trypanosomes and spirochetes found in the blood of owls are related to the leucocytozoon.

9. The trypanosomes which are at times present in ticks are not developmental forms of *Piroplasma*. It has already been shown that the trypanosomes in tsetse flies are not related to *Tr. gambiense* or *Tr. Brucei*.

10. The possibility of the trypanosomes of birds and other vertebrates developing in the gut of insects, while not excluded, has not been demonstrated. In the mosquito, *Tr. Lewisi* and *Tr. Brucei* lose their infectiousness, more or less rapidly, but the enfeebled organism may survive in the gut for 36–48 hours. The conditions

in the digestive tube of insects is not as favorable as in the test-tube, and this fact goes to show that insect carriers, such as tsetses, are mere passive hosts.

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## EXPLANATION OF PLATES.

The accompanying photographs are all taken at the uniform magnification of 1,500 diameters, and, as such, they are directly comparable with those of other cultural trypanosomes previously given. The preparations were stained by a modified Romanowsky method.

### PLATE 7. CRITHIDIA FASCICULATA.

(As found in mosquitoes.)

Fig. 1.—*Crithidia* from mosquito No. 99.

Fig. 2.—Same preparation, showing *Crithidia* and *Herpetomonas.* Note the square anterior end and the short flagellum of the former; the diplosome posterior to the nucleus in the latter.

Fig. 3.—Elongated *Crithidia* from the same preparation.

Fig. 4.—*Crithidia* from mosquito No. 56.

Fig. 5.—*Crithidia* from mosquito No. 46.

Fig. 6.—*Crithidia* from a culture nine days old (Gen. 1); compare Figs. 5 and 6; the former is derived from a mosquito, the latter from a culture. Note the similarity in size and structure, showing that the wild *Crithidia* are really cultural forms *in vivo*. Compare also with cultural forms shown on Plates 2 and 3.

### PLATE 8. CRITHIDIA FASCICULATA.

(Cultural forms of Strain No. 10.)

Figs. 1 and 2.—Multiplication rosettes, showing large and small cells. Note the unstained channel in some showing position of whips which are directed centrally; also the small round or oval blepharoplast adjoining the nucleus. Nine-day culture, Gen. 1.

Fig. 3.—Dividing form, final stage; showing two nuclei, two blepharoplasts, and two flagella, one of which is longer than that of the parent cell. Three-day culture, Gen. 39.

Fig. 4.—Part of a rosette of elongated *Crithidia* with flagella directed centrally; note the stippling, Gen. 32.

Fig. 5.—Group of short *Crithidia* from the same preparation as Figs. 1 and 2. Note the truncated anterior end.

Fig. 6.—Elongated *Crithidia* from the same preparation as preceding.

### PLATE 9. CRITHIDIA FASCICULATA.

(Cultural forms from Strain No. 10. Figs 1, 2, and 3 are from a preparation of the 20th generation or sub-culture.)

Fig. 1.—An agglutination group of *Crithidia.*

Fig. 2.—Short oval form showing square anterior end, blepharoplast adjoining the nucleus, and a short flagellum, bent within the cell.

FIG. 3.—Short, thick form with concave anterior end; blepharoplast and flagellum as before.

FIG. 4.—Goblet-shaped *Crithidia*. Note the square anterior end and the constriction posterior to the nucleus. Also the dark granules at posterior end. Compare with the constricted forms shown in Schaudinn's. Figs. 1*b*, 2*b*, and 4*b*. From a third generation, two days old.

FIG. 5.—Normal and goblet-shaped *Crithidia* from the same preparation as preceding.

### PLATE 10. TRYPANOSOMA (Herpetomonas) CULICIS.

(As found in mosquitoes; compare with cultural forms on Plate 12.)

FIG. 1.—Pale slender form of medium length; showing flagellum, blepharoplast, nucleus, and posterior diplosome or acrosome. From mosquito No. 52. Compare with cultural form shown in Fig. 1, Plate 12.

FIG. 2.—Longer form from same preparation; showing undulating membrane, flagellum, blepharoplast, nucleus, and posterior body. Compare with cultural form shown in Fig. 2, Plate 12.

FIG. 3.—From same preparation as preceding. Note the short new flagellum ( ?) indicating initial division.

FIG. 4.—A medium form, showing two diplosomes or pairs of posterior bodies. From mosquito No. 99.

FIG. 5.—A similar cell from mosquito No. 52, showing two diplosomes, and possibly a third against the nucleus.

FIG. 6.—Small form (male ?) from same preparation as preceding.

### PLATE 11. TRYPANOSOMA (Herpetomonas) CULICIS.

(As found in mosquitoes and in cultures.)

FIG. 1.—Rounded-up form from mosquito No. 4. Note the two large diplosomes between the blepharoplast and nucleus.

FIG. 2.—Short divisional form, showing two flagella, two blepharoplasts, two nuclei, and two diplosomes or pairs of posterior bodies. From mosquito No. 52.

FIG. 3.—Cultural form from Strain No. 29, Generation 17, showing early stage of division. Note the thickening of the base of flagellum which is due to the presence of a short new whip; division of the blepharoplast; undivided nucleus and the presence of two diplosomes or pairs of posterior bodies.

FIG. 4.—Divisional form from same preparation as preceding; showing two flagella and two nuclei with rather indistinct posterior bodies. Two blepharoplasts are present, but one lies partly over the other.

FIG. 5.—Divisional form from same preparation, showing two prominent blepharoplasts as well as two flagella and two nuclei.

FIG. 6.—Divisional form from same preparation, showing complete division; prominent posterior bodies and blepharoplasts.

### PLATE 12. TRYPANOSOMA (Herpetomonas) CULICIS.

(Cultural forms from the 17th generation of Strain No. 29.)

FIG. 1.—Pale, slender form, corresponding to that shown in Fig. 1, Plate 10. Note the prominent diplosome.

FIG. 2.—Longer form, showing undulating membrane and posterior diplosome. Compare with the mosquito form shown in Fig. 2, Plate 10.

FIG. 3.—Rounded-up form, showing flagellum, blepharoplast, nucleus, and prominent diplosome. Compare with Fig. 1, Plate 11.

FIG. 4.—Very short and long male and indifferent forms; also a rounded-up cell as in preceding figure, but showing two diplosomes or pairs of posterior bodies.

FIG. 5.—Large and rather rare form (female ?) with two nuclei. The diplosomes are in the anterior portion.

FIG. 6.—Large (female ?) form, similar to preceding, in process of division.

### PLATE 13. TRYPANOSOMA CHRISTOPHERSI

(From the dog-tick, *Rhipicephalus sanguineus*, in preparation of Captain S. R. Christophers, I. M. S.)

FIGS. 1, 2, AND 3.—Forms showing prominent undulating membrane and blepharoplast anterior or lateral to the nucleus. Note the sharp posterior end and the presence of globules.

FIG. 4.—The same, with less prominent undulating membrane.

FIG. 5.—Form showing rounding-up of posterior end, also large globules.

FIG. 6.—Unequal longitudinal division.

# PLATE 7.

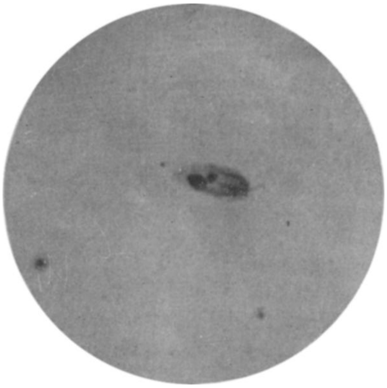
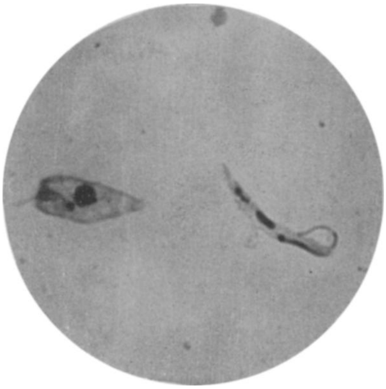


FIG. 1.



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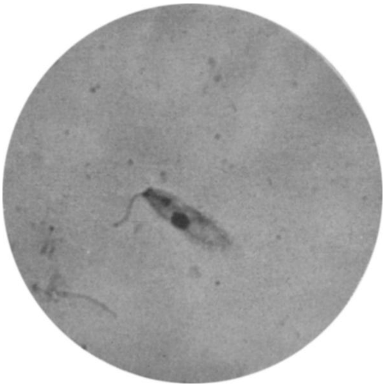

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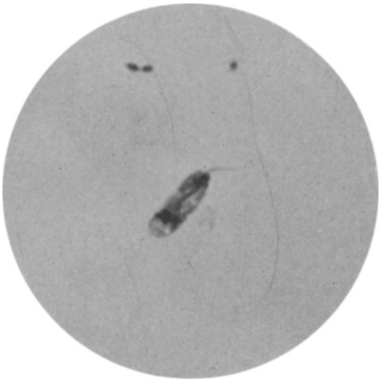

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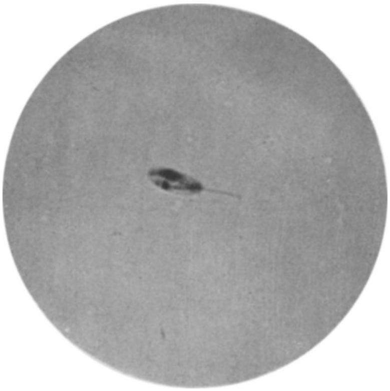

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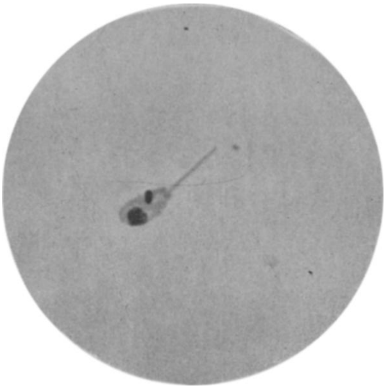


FIG. 6.

# PLATE 8.

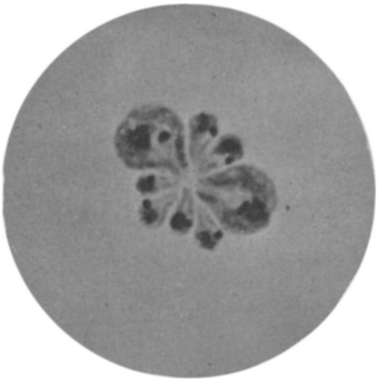
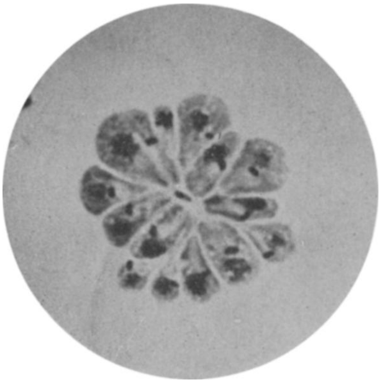


FIG. 1.



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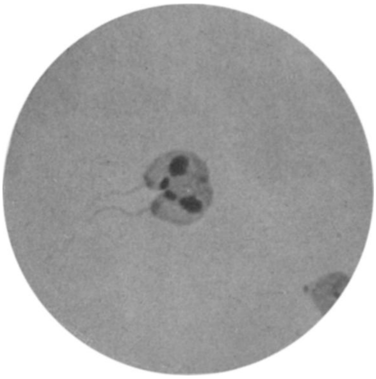

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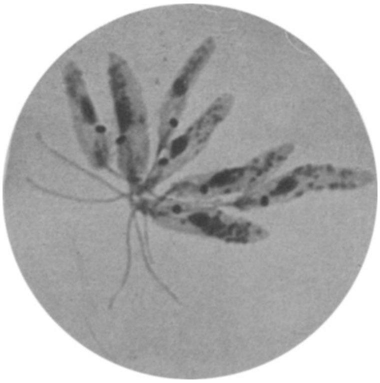

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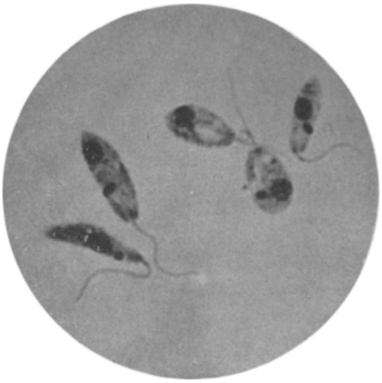

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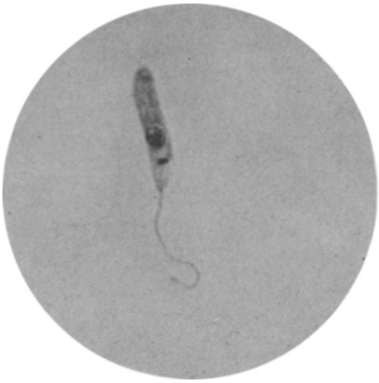


FIG. 6.

# PLATE 9.

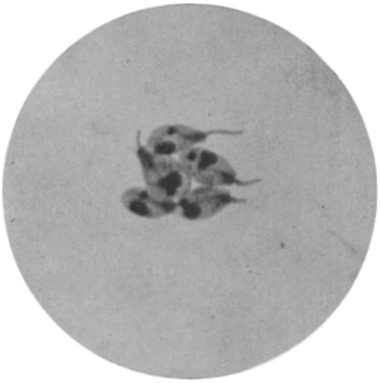


FIG. 1.

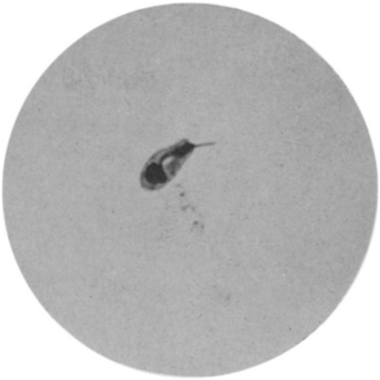
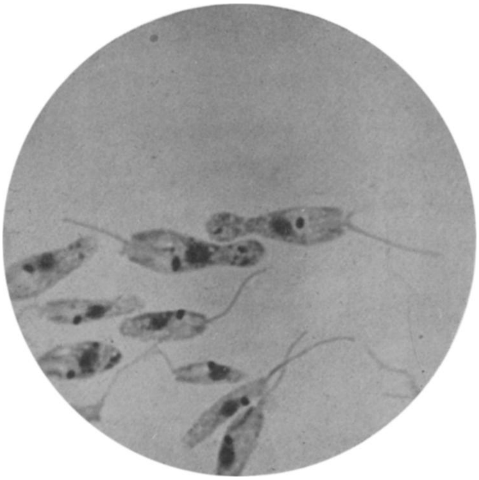


FIG. 2.



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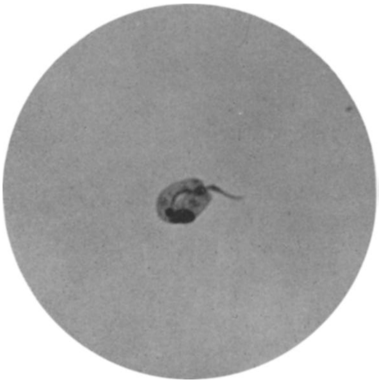

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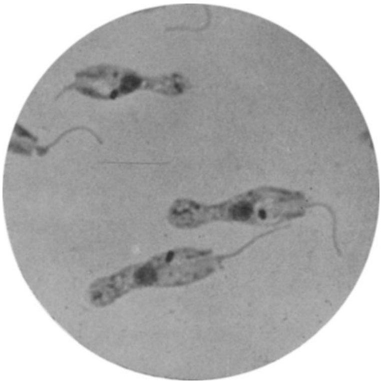
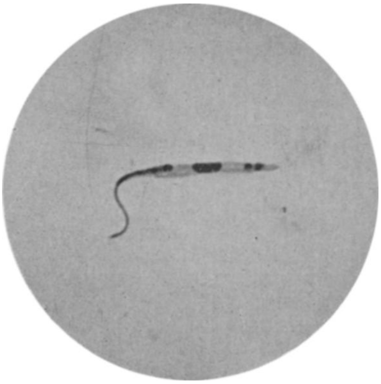


FIG. 4.

# PLATE 10.



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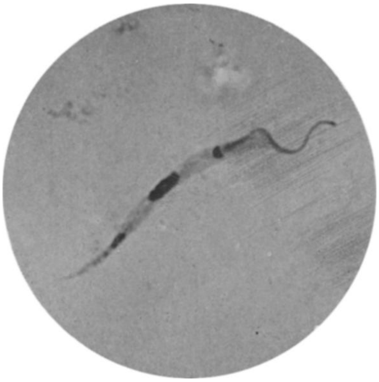

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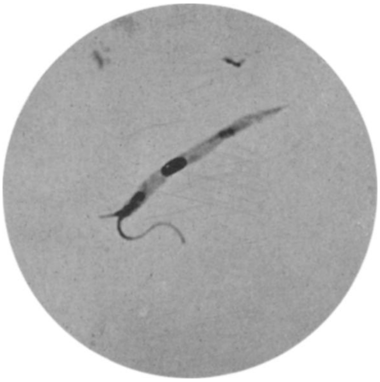

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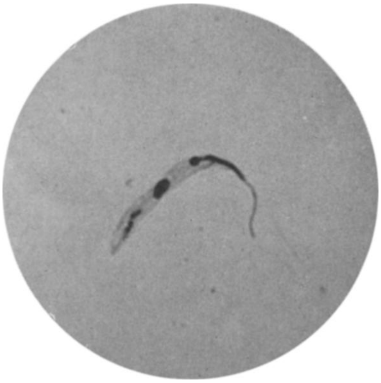

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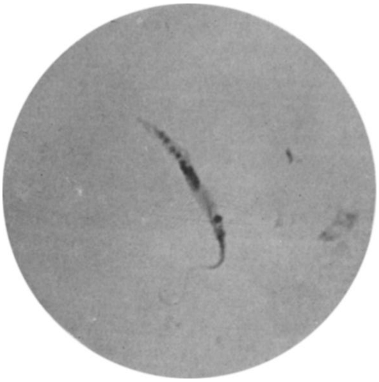

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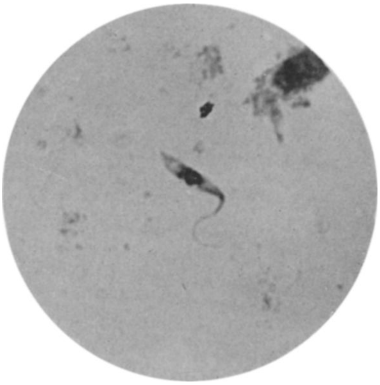


FIG. 6.

PLATE II.

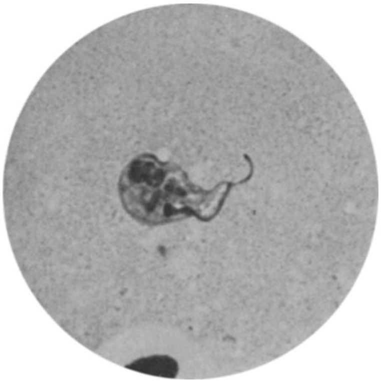
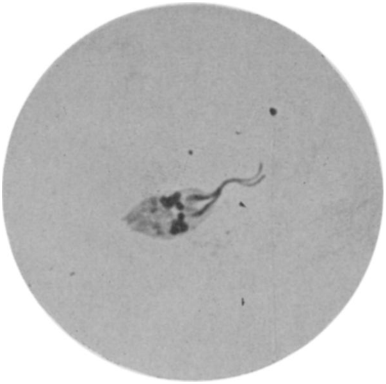


FIG. 1.



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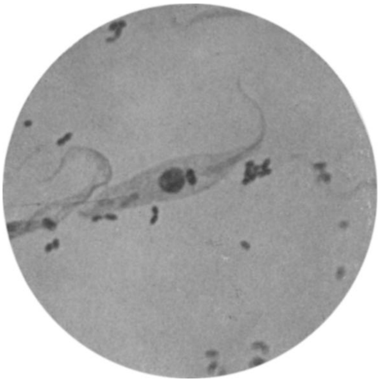

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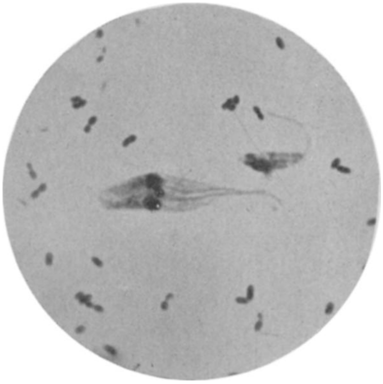

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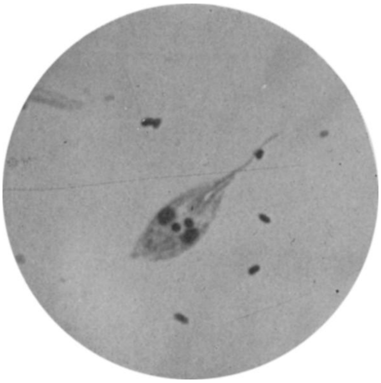

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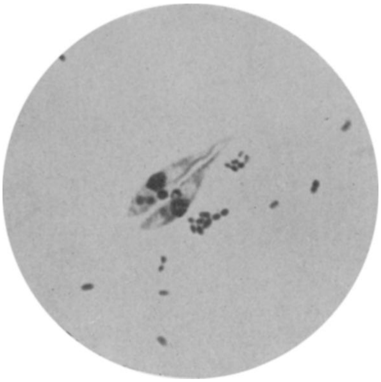
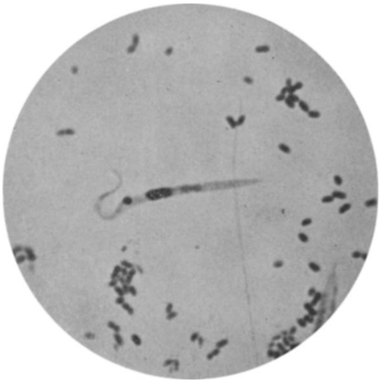


FIG. 6.

PLATE 12.



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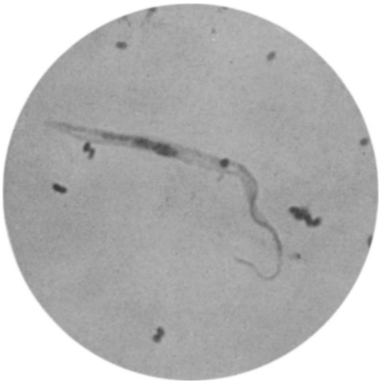

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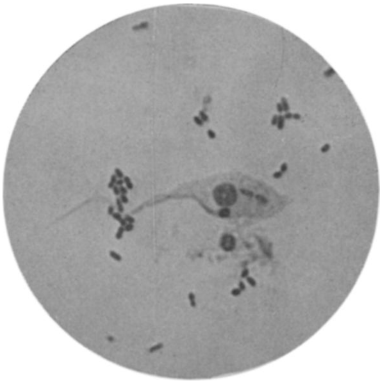

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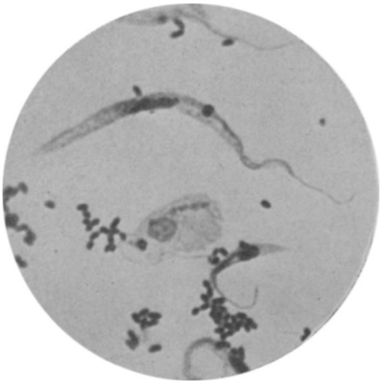

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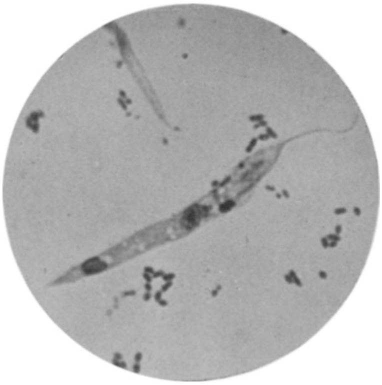

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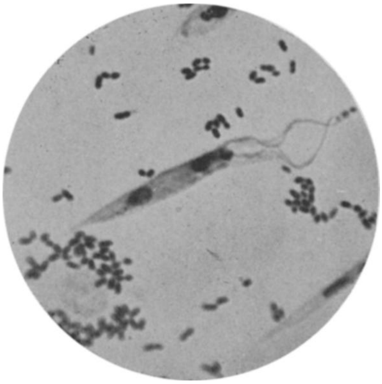
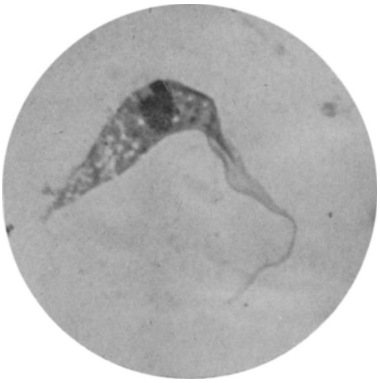


FIG. 6.

PLATE 13.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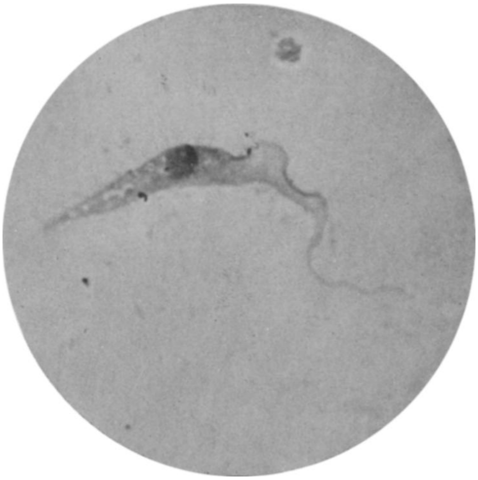
FIG. 2.

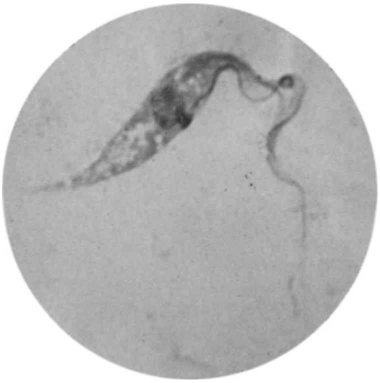
FIG. 3.

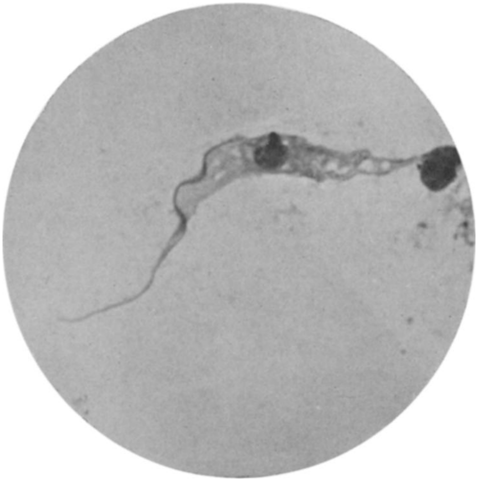
FIG. 4.

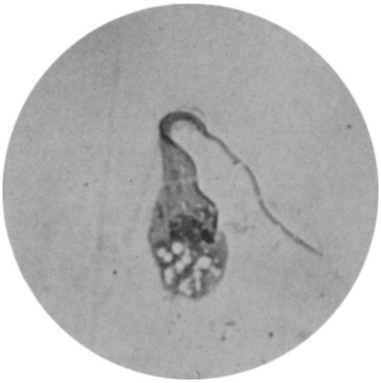
FIG. 5.

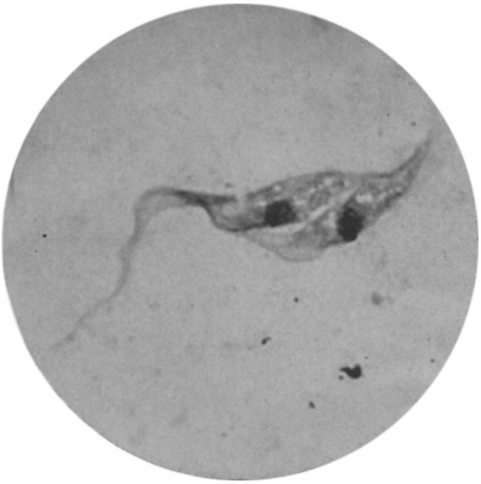
FIG. 6.